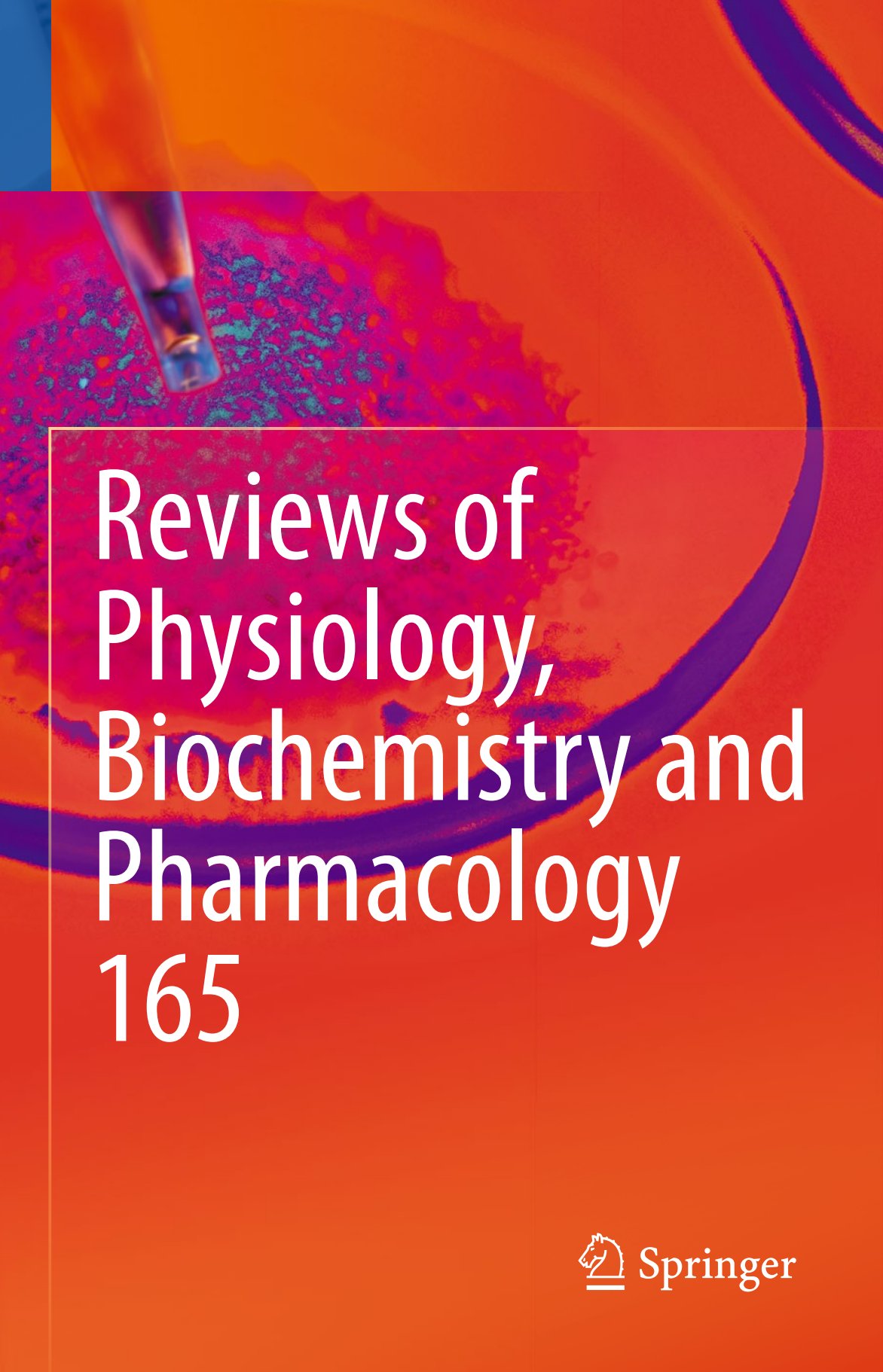
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Contents

The Exocrine Pancreas: The Acinar-Ductal Tango in Physiology and Pathophysiology

Peter Hegyi and Ole H. Petersen

Abstract There are many reviews of pancreatic acinar cell function and also of pancreatic duct function, but there is an almost total absence of synthetic reviews bringing the integrated functions of these two vitally and mutually interdependent cells together. This is what we have attempted to do in this chapter. In the first part, we review the normal integrated function of the acinar-ductal system, with particular emphasis on how regulation of one type of cell also influences the other cell type. In the second part, we review a range of pathological processes, particularly those involved in acute pancreatitis (AP), an often-fatal human disease in which the pancreas digests itself, in order to explore how malfunction of one of the cell types adversely affects the function of the other.

Contents

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Abbreviations

ACh	Acetylcholine
ASICs	Acid-sensing ion channels
AP	Acute pancreatitis
ATP	Adenosine-5′-triphosphate
UTP	Alpha-D-glucose-1-phosphate uridylyltransferase
CLCs	Ca^{2+}-activated Cl^- channels
CaM	Calmodulin
PRSS1	Cationic trypsinogen
CCK	Cholecystokinin
CTRC	Chymotrypsin C
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CF	Cystic fibrosis
DIDS	Diisothiocyanostilbene disulfonate
FAEEs	Fatty acid ethyl esters
FAs	Fatty acids
IP_3	Inositol triphosphate
$[Ca^{2+}]_i$	Intracellular Ca^{2+} level
IRK-8	Inwardly rectifying K^+ -8
P2X	Ionotropic purinoceptor
NHE	Na^+/H^+ exchangers
PSTI	Pancreatic secretory trypsin inhibitor
PMCA	Plasma membrane Ca^{2+} ATPase pump
KCNQ1	Potassium voltage-gated channel subfamily Q, member 1
PAR2	Protease-activated receptor 2
ROS	Reactive oxygen species
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
TLC	Taurolithocholic acid

TRPVs	Transient receptor potential ion channels of the vanilloid subtype
KCNKs	Two-pore domain potassium channels
TWIK-2	Two-pore domain weakly inward rectifying
ZGs	Zymogen granules

1 Introduction

The acinar-ductal functional units of the human exocrine pancreas secrete around 2 l/day of a solution rich in bicarbonate and digestive enzymes, which is essential for the normal digestive process (Argent 2006; Lee and Muallem 2008; Petersen 2008). Generally the acinar cells secrete the digestive enzymes with a small volume of a Na^+, Cl^-, and H^+-rich fluid (Petersen 2008), whereas the ductal cells secrete mainly Cl^- and HCO_3^- with a large volume of fluid (Argent 2006). This alkaline- and enzyme-rich pancreatic juice will then reach the duodenum where bicarbonate ions will neutralize the acidic chyme entering from the stomach and where the pancreatic enzymes will be activated (Argent 2006). This complex physiological process will secure the normal physiological digestion of the food. Unfortunately, research on the two principal cell types of the exocrine pancreas became to a large extent segregated in the second half of the last century, and in almost all cases the studies as well as reviews have been focused either on acinar or on duct cells. However, there are plenty of data suggesting the presence of coordinated secretion, which involve both autocrine regulatory mechanisms and cross talk between the acinar and ductal cells (Hegyi and Rakonczay 2010; Hegyi et al. 2011a, b) (Fig. 1).

Importantly, if the coordinated finely tuned secretion is damaged by either of the cell types, severe pancreatic damage may occur which will of course not be confined to the impaired cell type (Hegyi et al. 2011b). For example, impaired ductal fluid secretion and lower intraluminal pH which occurs in cystic fibrosis (CF) lead to acinar cell dysfunction too (Freedman et al. 2001), whereas mutations of cationic trypsinogen (PRSS1) (Whitcomb et al. 1996), trypsin inhibitors (SPINK1) (Witt et al. 2000), or chymotrypsin C (CTRC) (Rosendahl et al. 2008) can elevate the risk of (or lead to) chronic pancreatitis in which case the ductal bicarbonate secretion is also impaired.

In this chapter, we uniquely bring the acinar and ductal cells together and summarize our current knowledge of their communication paths in physiology and pathophysiology.

2 Physiological Aspects of the Acinar-Ductal Interactions

The pancreas has a “tree-and branch-like” structure with secretory endpieces in the form of acini connected to branches of small ducts merging into larger ducts finally connected to the main pancreatic duct. Acinar cells within individual acinar units

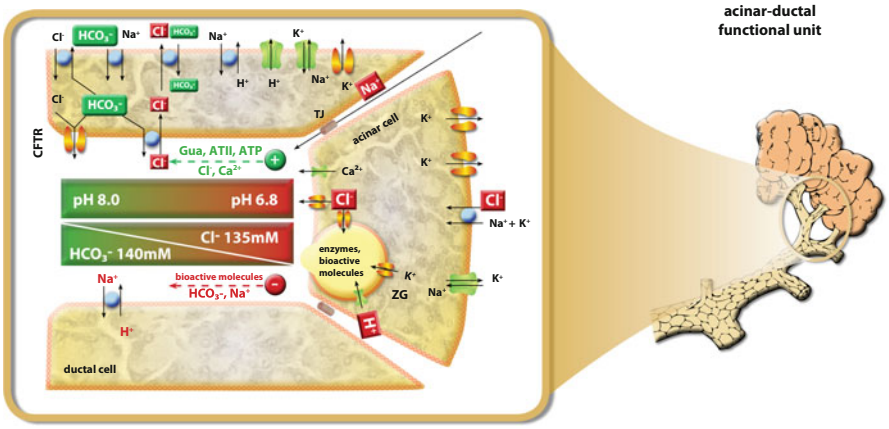

Fig. 1 *The acinar-ductal functional unit*. The *acinar cells* secrete an isotonic NaCl-rich fluid. Cl^- enters the acinar cells through basolateral Na^+-K^+-$2Cl^-$ cotransporters, which can operate due to the Ca^{2+}-activated K^+ channels and the Na^+/K^+ pump. Thus, the net transport across the basolateral membrane is uptake of Cl^-. Cl^- leaves the acinar cells via the Ca^{2+}-activated Cl^- channels in the apical (luminal) membrane. Whereas Cl^- is transported through the acinar cells, Na^+ moves from the interstitial fluid to the acinar lumen via the leaky tight junctions and water follows through both the tight (leaky) junctional pathway and through the cells [water movements not shown in order not to overcrowd the diagram]. (The secretion processes are activated by a rise in the cytosolic Ca^{2+} concentration evoked by stimulation of acetylcholine and/or cholecystokinin receptors on the basolateral acinar cell membrane, which results in the production of intracellular Ca^{2+}-releasing messengers [not shown in this figure]). The zymogen granules of the acinar cells contain hundreds of proteins and bioactive molecules including more than 20 different digestive (pro)enzymes. These molecules are stored in an acidic fluid composed by ion transport mechanisms shown in the figure. The *ductal cells* secrete an isotonic HCO_3^--rich fluid and this process is mediated by the basolateral and apical ion transport mechanisms indicated. (This process is stimulated by the hormone secretin, which evokes intracellular production of cyclic AMP [not shown in this figure]). Luminal Cl^- and Ca^{2+}, guanylin, angiotensin II, and ATP stimulate HCO_3^- efflux from ductal cells, whereas Na^+ and HCO_3^- in the lumen, and most probably some unknown bioactive molecules secreted by acinar cells, inhibit HCO_3^- influx. This coordinated finely tuned process will provide around 2 l/day of an isotonic solution rich in bicarbonate and digestive enzymes. *CFTR* cystic fibrosis transmembrane conductance regulator, *Gua* guanylin, *ATII* angiotensin II, *ATP* adenosine-5′-triphosphate, *ZG* zymogen granules, *TJ* tight (leaky) junctions

are electrically coupled via gap-junctional channels (Petersen and Findlay 1987). Ultrastructural studies show that the density of gap junctions between adjacent acinar cells is extremely high with the major part of the lateral plasma membranes dominated by these structures (Meda et al. 1983), which accounts for the complete electrical coupling (coupling ratio between adjacent cells = 1) between acinar cells within individual acinar units (Iwatsuki and Petersen 1978). On the other hand, no electrical coupling has been found between acinar cells in different neighboring acinar units, indicating the absence of gap-junctional channels between acinar and duct cells (Iwatsuki and Petersen 1978). There is also direct chemical coupling between acinar cells within individual acinar units as demonstrated by direct visualization of the movements of the fluorescent dyes, fluorescein or Lucifer

Yellow (mol. wt. 457) injected into one acinar cell to adjacent cells, eventually spreading to the whole acinar unit (Iwatsuki and Petersen 1979; Findlay and Petersen 1983). Lucifer Yellow injected into acinar cells was never detected in duct cells, confirming the absence of gap-junctional coupling between adjacent acinar and duct cells (Findlay and Petersen 1983). Sustained intra-acinar injection of Lucifer Yellow revealed a finite limit to the extent of dye spread and serial sections of pancreatic tissue fixed after complete dye spread allowed reconstruction of the whole acinar network, which turned out to consist of ~100 to 250 cells (Findlay and Petersen 1983) in reasonable agreement with the electrophysiological analysis indicating that there might be up to 500 well-coupled cells in individual acinar units (Iwatsuki and Petersen 1978). Acinar cells within individual acinar units can be acutely and reversibly uncoupled, both electrically and chemically, by supramaximal stimulation with acetylcholine (ACh) or cholecystokinin (CCK) (Iwatsuki and Petersen 1978; Petersen and Findlay 1987). The functional importance of the gap-junctional communication between acinar cells is not understood, but sharing of the relatively sparse functional innervations and/or sharing of the relatively low density of high-conductance Ca^{2+}-activated K^+ channels may be important (Petersen and Findlay 1987). The uncoupling observed in response to supramaximal stimulation is most likely a protective mechanism designed to isolate a cell that experiences a dangerously high level of cytosolic-free Ca^{2+} or a low intracellular pH (Petersen and Findlay 1987). The absence of direct coupling between acinar and duct cells may be functionally important as it allows separate fine regulation of these two cell types without messengers, for example, inositol triphosphate (IP_3) and cyclic adenosine monophosphate (cAMP) generated in one cell type necessarily invading the other type.

3 Composition of Pancreatic Juice Secreted by Acinar Cells to the Ductal Lumen

The neurotransmitter acetylcholine and the neuropeptide cholecystokinine stimulate the acinar cells to secrete an isotonic NaCl and protein-rich juice into the ductal system (Petersen 2008).

3.1 Pancreatic Digestive Enzymes and Other Proteins

The acinar cells secrete over 20 different enzymes including more than 10 different proteases (which is the major enzyme product of acinar cells), lipases, ribonucleases, amylases, and hydrolases (Keller and Allan 1967; Rinderknecht 1993; Whitcomb and Lowe 2007). They are generally synthesized and secreted in an inactive form (proenzymes) in order to avoid autodigestion of the organ. The

physiological pancreatic enzyme cascade starts only in the duodenum with the activation of trypsinogen by enteropeptidases; subsequently, the active trypsin will activate the other proenzymes. To avoid inappropriate intrapancreatic activation of proteases, pancreatic acinar cells secrete trypsin inhibitors (pancreatic secretory trypsin inhibitor [PSTI]) (Kazal et al. 1948) which are capable of trapping active trypsin. Adenosine-5′-triphosphate (ATP) was found to be co-released with enzymes in response to cholinergic and hormonal stimuli (Haanes and Novak 2010). ATP is transported via vesicular nucleotide transporters which can be stimulated by elevation of exogenous pH or Cl^- concentration (Haanes and Novak 2010). Recently, proteomic analyses of rat zymogen granules (ZGs) found 371 different proteins including enzymes, membrane proteins, juice proteins, transporters, and channels (Rindler et al. 2007). Although additional proteomics analysis confirmed the large quantity of bioactive molecules (Chen and Andrews 2008, 2009), their physiological roles need to be understood.

3.2 Ions

The acinar cells secrete little in the absence of nervous or hormonal stimulation, but are activated by ACh released from parasympathetic nerve endings surrounding the acini or by the hormone CCK. Both these physiological stimuli activate mechanisms that result in repetitive cytosolic Ca^{2+} spikes driving both acinar fluid and enzyme secretion. The intracellular Ca^{2+} signaling mechanisms have been extensively reviewed (Petersen 1992, 2005; Petersen and Tepikin 2008) and will therefore not be dealt with in any detail here.

The isotonic NaCl-rich fluid produced by the acinar cells in response to stimulation is the result of ion transports occurring across both the basolateral and apical plasma membranes. At the basolateral membrane, the crucial transport proteins are the Ca^{2+}- and voltage-activated K^+ channels (surprisingly not expressed in mouse or rat pancreatic acinar cells), but clearly present and important in, for example, pig and human pancreatic acinar cells (Petersen and Maruyama 1984; Petersen et al. 1985), the Na^+-K^+ pump and the Na^+-$2Cl^-$-K^+ cotransporter, functioning together as a Cl^- uptake mechanism, whereas at the apical (luminal) membrane the most important transporter is the Ca^{2+}-activated Cl^- channel (Petersen and Findlay 1987; Park et al. 2001). Cl^- thus passes through the acinar cells, whereas Na^+ moves from the interstitial fluid to the acinar lumen via the intercellular pathway through the leaky tight junctions (Petersen and Findlay 1987). The crucially important exclusive localization of the Ca^{2+}-activated Cl^- channels (CLCs) at the apical membrane has been demonstrated directly in studies combining high-resolution Ca^{2+} imaging and patch clamp current recording, in which it was shown that the Cl^- channels could only be activated by specifically uncaging caged Ca^{2+} near the apical, but not the basolateral membrane (Park et al. 2001).

The membrane of pancreatic acinar ZGs also contains several ion channels, which may or may not play a role in the formation of the fluid content secreted by

acinar cells into the ductal lumen. Protons are taken up into the ZG via a H^+-ATPase pump (Thevenod et al. 1989; Behrendorff et al. 2010) which is electrically balanced by influx of Cl^- (Pazoles and Pollard 1978; Gasser et al. 1988) via diisothiocyanostilbene disulfonate (DIDS)-sensitive CLC-2 or CLC-3 Cl^- channels (Thevenod 2002; Kelly et al. 2005). Inwardly rectifying K^+-8 (IRK-8) (Kelly et al. 2005), potassium voltage-gated channel, subfamily Q, member 1 (KCNQ1) (Braun and Thevenod 2000; Lee et al. 2008) and two-pore domain weakly inward rectifying (TWIK-2) (Rindler et al. 2007) K^+ channels were shown to be the major proteins driving K^+ inside the granule. The DIDS-sensitive Ca^{2+}-activated CLCA channel will drive HCO_3^- inside the granules which may promote exocytosis (Quinton 2001; Thevenod et al. 2003). Proteomic analyses suggested additional type of transporters such as Cl^-/K^+ cotransporter and Na^+/K^+ ATPase; however, their functional confirmation still needs to be done (Rindler et al. 2007).

Although it might be tempting to suggest that acinar fluid secretion is a consequence of the exocytotic insertion of ZG membrane into the apical plasma membrane, evidence from the (in many ways rather similar) parotid gland suggests that this is unlikely to be true. In the parotid gland, exocytotic enzyme secretion is principally stimulated by activation of beta-adrenergic receptors, whereas fluid secretion is mainly stimulated by activation of muscarinic and alpha-adrenergic receptors. It has been shown that beta-adrenergic stimulation hardly produces any conductance changes in the acinar membranes, whereas cholinergic and alpha-adrenergic stimulation massively increases the membrane conductance (Iwatsuki and Petersen 1981). When, during continuous beta-adrenergic stimulation – evoking a steady and high level of amylase secretion – cholinergic stimulation is applied, this results in a dramatic increase in acinar membrane conductance with only a very minor increase in amylase secretion (Iwatsuki and Petersen 1981). This is incompatible with the view that the conductance changes necessary for the acinar fluid secretion are due to insertion of granule membrane into the apical plasma membrane, but suggests strongly that fluid secretion is due to activation of ion channels in the plasma membrane entirely unconnected with the ZGs.

4 Sensing Mechanisms of the Ductal Cells

There are many molecules/ions secreted by the acinar cells that regulate the physiological process of pancreatic ductal bicarbonate secretion. In this chapter, we summarize the current knowledge of the effects of acinar fluid contents on ductal secretion and highlight future lines of research to understand the regulation of pancreatic fluid secretion.

4.1 Ions in the Pancreatic Juice Effecting Pancreatic Ductal Secretion

As we discussed above, pancreatic acinar cells secrete an isotonic juice including Na^+, Cl^-, H^+, Ca^{2+} into the pancreatic ducts (Petersen 2008). The ductal secretory processes are very much dependent on the ionic composition of the luminal fluid. Elevation of the luminal Cl^- and Ca^{2+} concentration stimulates HCO_3^- efflux via the Cl^-/HCO_3^- exchangers elevating the luminal HCO_3^- concentration, whereas elevation of the Na^+ concentration stimulates HCO_3^- influx thus decreasing the luminal HCO_3^- concentration in the pancreatic ductal tree. HCO_3^- in an autocrine manner dose dependently inhibits both the (cystic fibrosis transmembrane conductance regulator) CFTR Cl^- channel and the Cl^-/HCO_3^- exchangers.

4.2 Chloride and Bicarbonate

Elevation of the Cl^- concentration in the ductal lumen will stimulate the HCO_3^- secretion providing the anionic substrate of the Cl^-/HCO_3^- exchangers from the luminal side (Park et al. 2010). In addition, luminal Cl^- is also essential for the activity of CFTR Cl^- channel (Wright et al. 2004), which highlights the crucial importance of acinar Cl^- secretion. Generally, in physiological circumstances, the acinar fluid contains 25 mM HCO_3^- and 135 mM Cl^-, which will reach the apical membrane of the proximal duct where the Cl^-/HCO_3^- exchange starts (Park et al. 2010). By the time the fluid leaves the pancreas, the ratio has been turned around to 140 mM HCO_3^- and 20 mM Cl^-, due to the ductal activity of the CFTR Cl^- channel and the Cl^-/HCO_3^- exchangers (Park et al. 2010). It is important to highlight that the high HCO_3^- and the low Cl^- concentration will inhibit both the CFTR Cl^- channel and the Cl^-/HCO_3^- exchangers, thereby preventing bicarbonate reabsorption via the apical membrane of the duct cells (Wright et al. 2004; Park et al. 2010).

4.3 Sodium

Much less is known about the physiological importance of intraluminal Na^+. It has been shown that there are Na^+/H^+ exchangers (NHE2 and 3) (Marteau et al. 1995; Lee et al. 2000; Rakonczay et al. 2006) and Na^+/HCO_3^- cotransporters (Luo et al. 2001; Park et al. 2002) on the apical membrane of the ductal cells. Generally the activity of both transporter types will decrease the luminal HCO_3^- concentration, thus retrieving luminal bicarbonate. There is evidence suggesting that the epithelial electrolyte and water secretion at the cellular level is not only mediated by "stimulatory pathways" but also by "inhibitory pathways" (Hegyi et al. 2003;

Hegyi and Rakonczay 2007; Kemeny et al. 2011). Such inhibitory pathways may be important in terms of reducing the hydrostatic pressure within the ductal lumen (preventing leakage of enzymes into the parenchyma of the pancreas), and in terms of switching off pancreatic secretion after the digestion has finished (Aponte et al. 1989). Substance P (Hegyi et al. 2003, 2005b; Kemeny et al. 2011), basolaterally applied ATP (Ishiguro et al. 1999), and 5-hydroxytryptamine (Suzuki et al. 2001) have all been shown to inhibit secretion from isolated pancreatic ducts, but their effects on the apical sodium transporters are unknown.

4.4 Hydrogen

One of the more recent findings in the physiology of acinar fluid secretion is that acinar cells co-release H^+ during exocytosis causing a significant acidosis in the proximal tubes of the pancreatic ducts (Behrendorff et al. 2010). Physiological concentrations of the secretagogue CCK (10–20 pM) decrease the luminal pH up to 1 pH unit and cause short-lasting extracellular acidifications during exocytosis (Behrendorff et al. 2010). Then, the protons are quickly neutralized by the alkaline fluid secreted by the proximal ductal epithelia (Hegyi et al. 2011a). Importantly, a pathophysiologically relevant concentration of the CCK-analogue cerulein (100 nM) decreases the luminal pH up to 2 pH unit. This long-lasting extracellular acidification is followed by disruption of tight junctions (Behrendorff et al. 2010). Furthermore, a decrease of luminal pH speeds up the autoactivation of trypsinogen inside the lumen and decreases the HCO_3^- secretion of the ductal cells, which all together will promote inflammatory disease of the pancreas (Pallagi et al. 2011). In order to ensure the physiological homeostasis of the pancreas and to prevent such a proton-induced damage, the duct cells should have a physiological proton-sensing mechanism to restore luminal pH (Hegyi et al. 2011a).

Four main classes of acid-sensing ion channels have been identified in the gastrointestinal tract (Holzer 2007), namely, the acid-sensing ion channels (ASICs) (Page et al. 2005), the two-pore domain potassium channels (KCNKs) (Holzer 2003), the transient receptor potential ion channels of the vanilloid subtype (TRPVs) (Liddle 2007), and the ionotropic purinoceptor (P2X) (Henriksen and Novak 2003). Although the two latter ones have been identified in the pancreas, there is still no convincing evidence available to prove their involvement in the acid-mediated regulation of HCO_3^- secretion.

4.5 Calcium

A close parallelism can be observed between the calcium and protein concentration of the pancreatic juice during secretin and CCK stimulation (Goebell et al. 1973; Gullo et al. 1984). Generally, the calcium concentration can reach 1–3 mM Ca^{2+}

(Goebell et al. 1973; Gullo et al. 1984) and can act as a paracrine signaling molecule (Bruce et al. 1999; Racz et al. 2002). Ca^{2+}-sensing receptors have been identified on the apical membrane of the pancreatic ducts in the human pancreas and adenocarcinoma cell lines (Racz et al. 2002). Activation of this G-protein-coupled receptor increases HCO_3^- and fluid secretion (Bruce et al. 1999). Since 70 % of the proteins in the acinar fluid are proteases (Petersen 2008), this mechanism is crucially important in order to elevate the intraluminal pH which inhibits trypsinogen activation (Pallagi et al. 2011).

4.6 Molecules in the Pancreatic Juice Affecting Pancreatic Ductal Secretion

4.6.1 Guanylin/Uroguanylin

One of the best examples of an autocrine regulatory mechanism in pancreatic ducts is the guanylin-regulated electrolyte secretion. The 15-amino acid peptide guanylin is highly expressed in centroacinar and proximal ductal cells (Kulaksiz et al. 2001), whereas the 16-amino acid peptide uroguanylin, besides the above-mentioned places, is also expressed in the interlobular ducts (Kulaksiz and Cetin 2001). Both peptides are secreted into the ductal lumen and stimulate the guanylate cyclase-C receptor which will stimulate the CFTR Cl^- channel via elevation of the intracellular cyclic guanosine monophosphate (cGMP) level and activation of protein kinase II (Kulaksiz and Cetin 2001; Kulaksiz et al. 2001).

4.6.2 Angiotensin II

Angiotensin II can be detected in the pancreatic juice, and angiotensin I and II receptors can be found on the apical side of pancreatic ductal cells (Leung et al. 1997), suggesting a local regulatory effect of the oligopeptide hormone in a paracrine fashion. Since angiotensin was not detectable either in the ductal or acinar cells (Regoli et al. 2003), but in the glucagon-secreting islet cells (Regoli et al. 2003), it is more than likely that besides the acinar-ductal interaction there is an islet-ductal regulatory process. Activation of angiotensin I or II receptors elevates intracellular Ca^{2+} level $[Ca^{2+}]_i$ and cAMP level and therefore activates CFTR current stimulating HCO_3^- secretion (Leung et al. 1999; Tahmasebi et al. 1999; Lam and Leung 2002; Leung 2007). However, the physiological role of the islet-ductal regulatory process remains unclear.

4.6.3 Adenosine-5′-triphosphate

Adenosine-5′-triphosphate can be detected in nano-molar concentration range in human pancreatic juice (Kordas et al. 2004). CCK-8, but not secretin, was found to stimulate both ATP-consuming and ATP-generating enzyme secretions (Yegutkin et al. 2006). Luminal ATP was also shown to activate the G-protein-coupled P2Y and the ligand-gated ion channel P2X expressed on the apical membrane of duct cell (Luo et al. 1999; Henriksen and Novak 2003; Novak 2008). Alpha-D-glucose-1-phosphate uridylyltransferase (UTP) were found to stimulate pancreatic ductal HCO_3^- secretion via intracellular Ca^{2+} elevation (Ishiguro et al. 1999; Szucs et al. 2006).

It is clear that in parallel with the enzymes, the acinar cells secrete many substances that stimulate pancreatic ductal fluid and bicarbonate secretion. The main benefit of the ductal fluid secretion is to decrease the transit time of the pancreatic proenzymes in the ductal tree, whereas, the very important physiological role of HCO_3^- is to preserve trypsinogen in its inactive form in the ductal lumen. This acinar-ductal coordinated secretion is absolutely essential in order to prevent the pancreas from being digested by its own enzymes. Therefore, it is not surprising that derangement of this process can lead to pancreatic inflammatory diseases.

5 Pathophysiological Aspects of Acinar-Ductal Interactions

The most important diseases of the exocrine pancreas are pancreatitis and CF. Acute pancreatitis (AP) is initiated by a pathological increase in protease activity inside the acinar cells, which causes autodigestion and ultimately is capable of destroying the acinar cells, the whole pancreas, and the surrounding tissue (Petersen and Sutton 2006). Repeated attacks of AP leads to chronic pancreatitis, where the normal-functioning exocrine cells are replaced by a fibrous matrix, which is cancer promoting (Petersen et al. 2011a). In CF, pancreatic HCO_3^- secretion in the ducts is impaired resulting in blockage of the ducts, which in turn destroys the acinar cells and results in failure to secrete adequate amounts of digestive enzymes. In both cases, the physiologically important normal interaction between acinar and duct cells is disrupted. In the human pancreas, it has been shown that primary ductal blockade leads to intra-acinar trypsinogen activation (Murphy et al. 2008) demonstrating directly the importance of duct function for preservation of normal acinar cell function (Fig. 2).

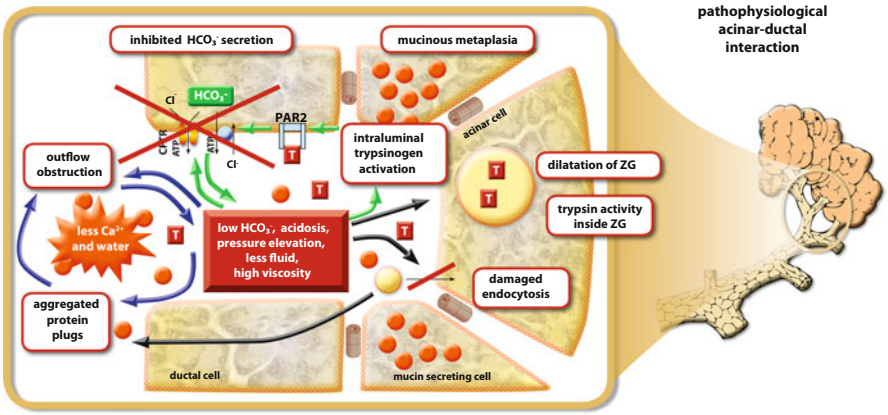

Fig. 2 *Pathophysiological aspects of acinar-ductal interactions.* Pancreatitis-inducing factors evoke Goblet cell (mucin-secreting cell) metaplasia as a replacement of the ductular and sometimes the acinar epithelium. This is often seen in chronic pancreatitis. Parallel to the metaplasia, the toxic agents also decrease HCO_3^- as well as fluid secretion by ductal cells, by which mechanism the formation of aggregated protein plugs is promoted. The protein plug will obstruct the outflow of pancreatic fluid, which will lead to the elevation of luminal pressure which further decreases HCO_3^- secretion and inhibits the outflow of enzymes from the ductal tree. Ductal blockade causes trypsinogen activation inside acinar cells. The decrease of luminal pH will strongly enhance autoactivation of luminal trypsinogen, which will inhibit CFTR channels and anion exchanger via luminal PAR2. This will further elevate the viscosity of pancreatic juice. Moreover, luminal acidosis will cause ZG dilatation and damage acinar endocytosis, which further elevates the protein concentration of the luminal content. These vicious cycles are crucial driving forces of the irreversible progressive chronic inflammation of the pancreas. *CFTR* cystic fibrosis transmembrane conductance regulator, *ATP* adenosine-5′-triphosphate, *ZG* zymogen granules, *TJ* tight junction, *T* trypsin, *PAR2* protease-activated receptor 2

6 Pathophysiological Changes in the Composition of Ions and Other Molecules in the Pancreatic Juice

6.1 Development of Protein Gel in the Ductal Lumen

One of the leading histological features of chronic pancreatitis is the formation of a protein gel in the fine pancreatic ducts (Sarles et al. 1965). Importantly this morphological observation is visible without damage of acinar cells or malformation of the duct of Wirsung (Sarles et al. 1965), suggesting that this mechanism may be responsible not only for the progression but also for the initiation of chronic pancreatitis. Ductal mucinous hyperplasia can be seen in 62 % of the patients suffering from chronic pancreatitis (Allen-Mersh 1985). In addition, Goblet-cell metaplasia is described as a replacement of the ductular and sometimes the acinar epithelium (Walters 1965). Despite the increase in the number of mucin-secreting cells, diminished HCO_3^- secretion is also a well known and very early defect in chronic pancreatitis (Braganza and Rao 1978; Freedman 1998). Therefore, the

viscosity of pancreatic juice is elevated due to the elevation of the mucin concentration in the ductal lumen and to the decreased fluid and bicarbonate secretion by ductal cells (Ko et al. 2012). Since HCO_3^- is essential for mucin swelling and hydration, by reducing Ca^{2+} cross-linking in mucins, the low concentration of HCO_3^- and high concentration of mucin will promote the formation of aggregated protein plugs (Chen et al. 2010). The protein plug will obstruct the outflow of pancreatic fluid, which will lead to higher pressure in the proximal direction from the plug. The elevated pressure will further decrease HCO_3^- secretion and inhibit the outflow of enzymes from the ductal tree (Suzuki et al. 2001). The decrease of luminal pH will strongly enhance the autoactivation of trypsinogen, which will inhibit CFTR Cl^- channels and anion exchanger via luminal protease-activated receptor 2 (PAR2) (Pallagi et al. 2011). This will further elevate the viscosity of pancreatic juice. This vicious cycle is one of the main driving forces of the irreversible progressive chronic inflammation of the pancreas. Importantly, the R122H mutation in the cationic trypsin causes the same inhibition of bicarbonate secretion as the normal trypsin via the PAR2 receptor (Ko et al. 2012). Since the autoactivation of trypsinogen is more enhanced in the "gain of function" mutations of PRSS1 (Sahin-Toth and Toth 2000; Simon et al. 2002), this process can be even more important in hereditary pancreatitis.

6.2 Changes in Luminal pH (pH_L)

Physiologically, as discussed in our earlier chapter, the luminal pH of pancreatic ducts depends on both acinar and ductal cell functions. Acinar cells can decrease the luminal pH down to 6.8 by secreting protons (Bhoomagoud et al. 2009), whereas pancreatic ductal cells can elevate the pH up to around 8.0 (Argent 2006). However, in pathophysiological states, pH_L can be decreased either due to enhanced proton secretion from the proximal part (Bhoomagoud et al. 2009) or bile reflux (Opie 1901) or injection of a contrast solution from the distal part of the duct (Noble et al. 2008). pH_L can also be decreased by decreased pancreatic ductal secretion (Wang et al. 2006; Hegyi et al. 2011b) and/or inflammation (Toyama et al. 1997; Patel et al. 1999). Noble et al. (2008) showed very elegantly that pH dropping below 7.0 causes pancreatic inflammation, whereas elevation – that is, correction – of the acidic luminal pH is beneficial (Freedman et al. 2001; Ko et al. 2010). Overall, irrespective of the manner by which luminal acidosis occurs, pH decrease is harmful, whereas pH elevation is beneficial for the pancreas.

6.3 *The Effects of the Luminal Fluid Changes on Acinar Cells*

The drop in luminal pH may not only induce or exacerbate pancreatic inflammation but also impair the physiological secretory function of the acinar cells. Glycoprotein 2 (GP2) release is around four times higher at pH 8.3 than 6.0 suggesting that bicarbonate secretion (alkalization of pH_L) regulates enzymatic cleavage of GP2, which is one of the major ZG membrane proteins in pancreatic acinar cells (Freedman et al. 1994, 1998a). Since amylase secretion (representing exocytosis) is only 20 % higher, whereas horseradish peroxidase uptake (representing endocytosis) is around eight times higher at pH 8.3 than at pH 6.0 (Freedman et al. 1998a), this suggests that endocytosis is much more strongly affected by pH_L than exocytosis. When ductal elements were isolated together with acinar cells, the anion exchanger DIDS inhibited GP2 as well as enzyme release from the acinar cells, clearly suggesting that bicarbonate secretion is essential for regulated enzyme secretion (Freedman et al. 1994). Besides the damage of endocytosis, acinar luminal dilatation with a marked reduction of ZGs can also be observed at pH 6.0 (Freedman et al. 1998a, b, 2001), which can be reversed by elevating the pH up to 8.3 (Scheele et al. 1996).

7 The Effects of the Main Pancreatitis-Inducing Factors on Acinar and Ductal Cells

Besides some genetic alterations, almost all cases of pancreatitis are due to a *stress/toxic factor*, which initiates pancreatic damage. In AP, 80–90 % of the stress is induced either by excessive ethanol consumption or by bile stones obstructing the outflow of pancreatic fluid, which besides the elevation of intraductal pressure may result in bile reflux into the ductal tree (Topazian and Pandol 2009). The remaining 10–20 % of the stress is shared between iatrogen, metabolic, infectious, neoplastic, and traumatic factors. Concerning chronic pancreatitis, 70 % of the cases are caused by alcohol, whereas, the rest is developed mostly due to other metabolic (e.g., hyperlipidemia, hypercalcemia), obstructive or autoimmune stress (Owyang and MJ 2009). Most recently, smoking was also shown to be an independent and dose-dependent factor for both acute and chronic pancreatitis (Alexandre et al. 2011). Generally, the pancreatitis-inducing factors in low concentration cause oscillatory calcium elevation and stimulate ductal bicarbonate and fluid secretion representing a ductal-defense mechanism. The stimulated secretion elevates the HCO_3^- concentration, that is, inhibits autoactivation of trypsinogen and washes out the toxic factors/enzymes from the pancreas. However if the toxic factors reach the cells in high concentrations, they will induce toxic sustained calcium signals (Voronina et al. 2002; Venglovecz et al. 2008), mitochondrial damage (Mukherjee et al. 2008; Maleth et al. 2011) with a consequent ATP depletion both in acinar and ductal cells.

This results in inhibited bicarbonate and fluid secretion in ductal cells (Hegyi et al. 2011b), intra-acinar and intraductal trypsinogen activation (Sherwood et al. 2007; Pallagi et al. 2011), ER stress (Kubisch and Logsdon 2008), and secretory block (Thrower et al. 2010).

8 The Effects of Pancreatitis-Inducing Factors on Ductal Cells

8.1 The Effects of Bile Acids on Ductal Cells

The effects of bile acids on the pancreatic ductal tree depend on the bile concentration and may be different in different parts of the duct system (Venglovecz et al. 2012). Concerning the main pancreatic ducts, bile acids in a concentration above 15 mM cause cell death with a consequent disruption of ductal integrity (Armstrong et al. 1985). Bile acids in concentrations between 2 and 15 mM are also toxic. They make the ducts permeable to molecules above 20,000 Da (Farmer et al. 1984; Armstrong et al. 1985), whereas normally the ducts are impermeable to molecules above 3,000 Da. Therefore, bile acids elevate the permeability of the epithelial barrier making the pancreas more vulnerable. Moreover, bile acids in these concentrations also elevate the permeability of the main duct to both Cl^- and HCO_3^- (Reber et al. 1979; Reber and Mosley 1980) causing loss of HCO_3^- concentration in the main duct. Bile acids in a concentration less than 2 mM have stimulatory effects both on Cl^- and K^+ conductances via Ca^{2+}-dependent mechanism.

Concerning the intra-/interlobular ducts, the same stimulatory and inhibitory pattern can be observed; however, these ducts are much more sensitive to bile than the main duct. In addition, there is a big difference between the effects of nonconjugated and conjugated bile acids. The conjugated bile acids have no effects on the secretory function of ductal cells at or below 1 mM concentration; however, the nonconjugated ones cause dual effects. Nonconjugated bile acids at a concentration of 100 μM stimulate bicarbonate secretion in a Ca^{2+}-dependent manner (Venglovecz et al. 2008; Venglovecz et al. 2011a), whereas at 1 mM concentration they damage the mitochondria (Maleth et al. 2013), deplete intracellular ATP, and block both the basolateral and apical ion transport mechanisms (Venglovecz et al. 2008; Ignath et al. 2009; Maleth et al. 2011).

8.2 The Effects of Ethanol and Its Metabolites on Ductal Cells

There is much less data available concerning the effects of ethanol and their metabolites on pancreatic ductal cells. Yamamoto et al. (2003) showed that ethanol in low concentration augmented the stimulatory effect of secretin, whereas in high concentration they inhibited the secretory rate (Yamamoto et al. 2003). We have recently investigated the effects of ethanol, fatty acids (FAs), fatty acid esters, and acetaldehyde on pancreatic ductal secretion (Venglovecz et al. 2011b; Hegyi et al. 2012). Our preliminary experiments showed that ethanol at a low concentration (10 mM) stimulates, whereas at a high concentration it inhibits ductal HCO_3^- concentration. Acetaldehyde has no effects, but both fatty acids (FAs) and fatty acid ethyl esters (FAEEs) strongly inhibit the CFTR Cl^- channel and the Cl^-/HCO_3^- exchanger (Venglovecz et al. 2011b; Hegyi et al. 2012). However, these intracellular signaling pathways need further investigation.

8.3 The Effects of Virus Infection on Ductal Cells

Viruses have been shown to induce AP. We have shown that intact pancreatic ducts can be infected with pseudorabies virus which is an alpha-herpesvirus (Hegyi et al. 2005a). The virulent strain of the virus was able to stimulate pancreatic bicarbonate secretion around four- to fivefold, suggesting a washout defense mechanism of the ductal epithelia.

9 The Effects of Pancreatitis-Inducing Factors on Acinar Cells

The key challenge in understanding the initiation of pancreatitis is to unravel the chain of intracellular events following exposure of the acinar cells to agents known to be instrumental in provoking an attack of AP, namely, bile acids, alcohol, FAs, and products of alcohol and FAs (Petersen et al. 2011a). The end point is the inappropriate intracellular protease activation causing necrosis, but there is still much uncertainty about the intermediary steps.

9.1 The Effects of Bile Acids on Acinar Cells

Bile acids can reach acinar cells either from the basolateral side or, if the ductal secretion is damaged, from the luminal side. The first important step in

understanding the primary action of bile acids on the acinar cells was the finding that these substances evoke increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), due to primary release from intracellular stores and subsequent activation of Ca^{2+} entry (Voronina et al. 2002). Further studies revealed that Ca^{2+} was released from both the endoplasmic reticulum (ER) and acid stores (Gerasimenko et al. 2006). The Ca^{2+} release occurs through both IP_3 and ryanodine receptors (Voronina et al. 2002; Gerasimenko et al. 2006; Petersen et al. 2011b). Bile acid concentrations that are easily within a pathophysiologically relevant range can evoke sustained global $[Ca^{2+}]_i$ elevations (Voronina et al. 2002; Gerasimenko et al. 2006) and cause Ca^{2+}-dependent cell death (Kim et al. 2002). Bile acids evoke mitochondrial depolarization (Voronina et al. 2004) and a reduction in the intracellular ATP level (Voronina et al. 2010), which causes inhibition of both sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and plasma membrane Ca^{2+} ATPase pump (PMCA) creating a vicious circle driving the cell to further damage (Barrow et al. 2008). The intracellular ATP level determines whether cell death occurs by apoptosis or necrosis. This has been demonstrated in patch clamp whole cell recording experiments, in which direct supply of ATP to the cell interior converts bile acid–induced necrosis to apoptosis (Booth et al. 2011). Patch clamp experiments revealed that bile acids also induce a Ca^{2+}-independent cationic current, even at a stimulus intensity not evoking any major increase in the intracellular Ca^{2+} level (Voronina et al. 2004), but the significance of this phenomenon is currently unclear. Some of the bile acid effects may be mediated via G-protein-coupled cell surface bile acid receptors as they were found to play a role in the initiation of intra-acinar Ca^{2+} elevation and zymogen activation (Perides et al. 2010). Pancreatic acinar calcineurin may also play a role in the development of the Ca^{2+} elevation suggesting that calcineurin inhibition may be a therapeutical possibility against bile-induced acinar cell damage (Muili et al. 2013). Notably, bile acids also increase intracellular and mitochondrial reactive oxygen species (ROS) concentrations promoting acinar cell apoptosis (Booth et al. 2011) suggesting a possible defense mechanism against the more severe cell necrosis. However, it should be noted that oxidant stress imposed on top of a steady small elevation of $[Ca^{2+}]_i$ can induce a significant amount of necrosis (Ferdek et al. 2012). Proteome analyses of AR42J cells showed that taurolithocholic acid (TLC) induced upregulation of 23 and downregulation of 16 proteins suggesting further mechanisms involved in the toxicity of bile acids on acinar cells (Li et al. 2012). Besides the effects of bile acids on ductal and acinar cells, experiments on Toll-like receptor 4-deficient mice suggest that leukocytes are also involved in the bile-induced pancreatic damage. The bile-induced pancreatic damage and pancreatic and lung myeloperoxidase activities were decreased in the receptor-deficient animals (Awla et al. 2011). Overall, the effects of bile acids on pancreatic acinar cells are complex and at this stage it is still difficult to judge the relative importance of several of the processes that have been indicated by often very different experimental protocols. It is, however, abundantly clear that excessive intracellular Ca^{2+} release, and therefore intracellular Ca^{2+} toxicity, is the central feature of the pathological bile actions, in both the ducts and the acini (Fig. 3).

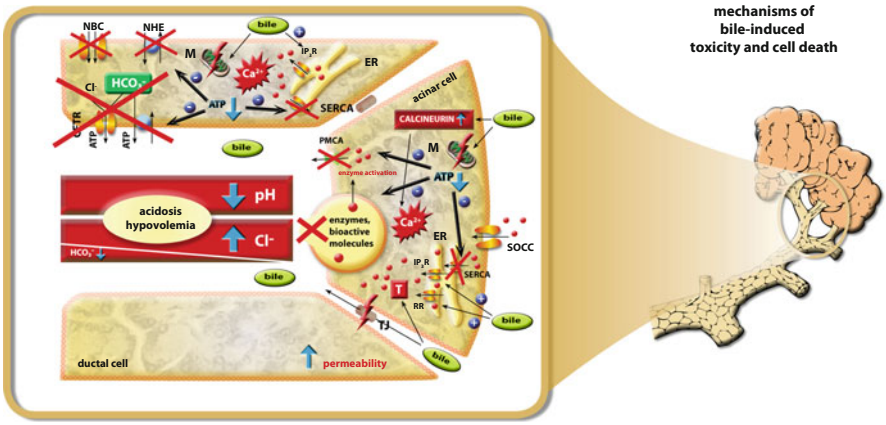

Fig. 3 *Mechanisms of bile-induced toxicity and cell death*. Bile acids in high concentration elevate the intracellular Ca^{2+} concentration via several mechanisms in both cell types. Bile acids enhance the activity of both IP_3R and RR which will release Ca^{2+} from the ER (and acid stores [not shown]) to the cytosol. This damages mitochondria and consequently decreases ATP production, so that neither SERCA nor PMCA can remove Ca^{2+} from the cytosol. This will not only maintain a dangerously high intracellular Ca^{2+} level, but due to the empty intracellular Ca^{2+} stores there is activation of SOCC. The elevated $[Ca^{2+}]_i$ and decreased ATP level will inhibit the exocytosis, cause intracellular trypsin activation in acinar cells, and also inhibit bicarbonate and fluid secretion from ductal cells. The activated enzymes will be stuck inside the pancreas and furthermore the damaged tight junctions will let the activated enzyme-rich solution flow into the interstitial space. All these processes will contribute to necrosis. *CFTR* cystic fibrosis transmembrane conductance regulator, *M* mitochondria, *ER* endoplasmic reticulum, *SOCC* store-operated Ca^{2+} channels, *SERCA* sarco/endoplasmic reticulum Ca^{2+}-ATPase, *PMCA* plasma membrane Ca^{2+} ATPase, *ATP* adenosine-5′-triphosphate, *ZG* zymogen granules, *TJ* tight junction, *T* trypsin

9.2 *The Effects of Ethanol, Fatty Acids, and Fatty Acid Ethyl Esters on Acinar Cells*

The effects of ethanol, and products of ethanol and fatty acids, are of considerable pathophysiological importance since they are major inducers of AP and also particularly effective in promoting chronic pancreatitis and thereby pancreatic cancer (Fig. 4).

Although the risk of developing pancreatitis increases with increasing alcohol intake, it is nevertheless the case that only a minority of heavy drinkers develop pancreatitis. In fact, alcohol (ethanol) itself mostly only exerts modest effects on acinar cell Ca^{2+} homeostasis, even in very high concentrations, whereas a combination of alcohol and FAs (FAEEs) causes massive intracellular Ca^{2+} release and acute intracellular trypsin activation (Criddle et al. 2004, 2006; Petersen and Sutton 2006; Gerasimenko et al. 2009). Fatty acids are taken up into cells via specific transporters (Doege and Stahl 2006), whereas ethanol diffuses very easily across the plasma membrane. Inside the acinar cells, FAEE synthases can catalyze the formation of FAEEs (Petersen et al. 2009). The control of these synthases is poorly

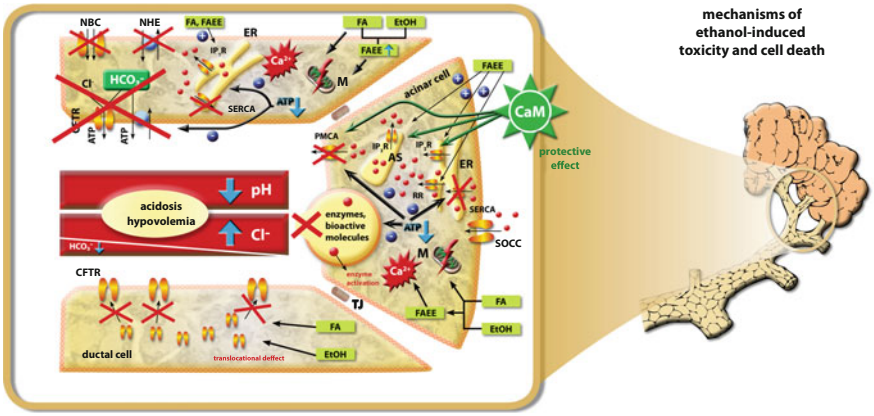

Fig. 4 *Mechanisms of toxicity and cell death induced by ethanol, fatty acids and fatty acid ethyl esters*. Fatty acid ethyl esters (FAEEs) are the most powerful releasers of Ca^{2+} from both the endoplasmic reticulum (ER) and acid stores. Ethanol itself can also release Ca^{2+} from intracellular stores, but is relatively weaker and in intact cells the protective effect of calmodulin(CaM) largely prevents ethanol itself from having much of a Ca^{2+} releasing effect. Fatty acids (FAs) increase the cytosolic Ca^{2+} concentration indirectly by inhibiting mitochondrial function and therefore adenosine-5′-triphosphate (ATP) production, so that Ca^{2+} pumps in both the ER and the plasma membrane cannot function properly. All these events contribute to stopping normal secretion in both acinar and duct cells as well as causing intra-acinar trypsinogen activation and necrosis

understood, but may be very important for understanding why some people develop pancreatitis after an alcoholic binge and others not. It is well known that the normal pathway for eliminating ethanol is by breakdown to acetylaldehyde, a process catalyzed by the enzyme alcohol dehydrogenase. As this is an oxidative process, it cannot occur in hypoxic cells, whereas the formation of FAEEs can. FAEEs are therefore also described as non-oxidative ethanol metabolites (Laposata and Lange 1986; Werner et al. 1997; Criddle et al. 2004, 2006).

FAEE-elicited Ca^{2+} release occurs from both the endoplasmic reticulum and acid stores in the granular part of the cells (mostly ZGs), but it has been shown that it is the Ca^{2+} liberation from the acid stores that is most directly linked to the increase in intracellular trypsin activity (Gerasimenko et al. 2009). The Ca^{2+} liberation occurs through IP_3 receptors of types 2 and 3 and deletion of the genes for these receptors markedly reduces, but does not abolish, the Ca^{2+} release from the acid stores as well as the trypsinogen activation induced by the FAEEs (Gerasimenko et al. 2009). The gene knockout experiments suggest that although the IP_3 receptors of types 2 and 3 constitute the most important pathway for FAEE-induced Ca^{2+} release, some of the Ca^{2+} must pass through other channels. Pharmacological experiments indicate that ryanodine receptors also have a role to play (Gerasimenko et al. 2009).

Although the effects of ethanol itself on intact acinar cells were very modest (Criddle et al. 2004), subsequent investigations on permeabilized cells revealed that low ethanol concentrations, at levels easily attained in the blood following

moderate intake of alcohol, could cause substantial liberation of stored Ca^{2+} (Gerasimenko et al. 2011). This release, as was the case with the FAAEs, occurred from both the endoplasmic reticulum and the acid stores in the granular area and caused significant increases in intracellular trypsin activity. Also in this case, it turned out that the major pathway for the Ca^{2+} release was through IP_3 receptors of types 2 and 3 (Gerasimenko et al. 2011). What is the reason for the major discrepancy between the results on intact and permeabilized acinar cells? One attractive and testable hypothesis was that in the permeabilized cells one or more components could have been washed out of the cell that normally inhibits Ca^{2+} release through IP_3 receptors. A small soluble protein like calmodulin (CaM) was a possible candidate and subsequent experiments in which CaM was added to the external solution surrounding the permeabilized acinar cells demonstrated that a physiologically relevant concentration of CaM did indeed markedly inhibit ethanol-induced intracellular Ca^{2+} release (Gerasimenko et al. 2011). It would therefore appear that acinar cells possess an intrinsic protective protein, namely, CaM, in a sufficiently high concentration to provide significant protection against the potentially toxic action of ethanol (Gerasimenko et al. 2011; Petersen et al. 2011a). If the intrinsic protective effect of CaM could be boosted by some agent, this might in principle be a useful step in preventing or reducing the severity of an attack of AP. Membrane-permeable Ca^{2+}-like peptides have been synthesized that can act as CaM activators, and it was recently shown that the ability of even a high ethanol concentration to increase trypsin activity was virtually blocked by the Ca^{2+}-like peptide-3 (CALP-3) (Gerasimenko et al. 2011).

It is known that high plasma concentrations of long-chain free fatty acids markedly increase the risk of pancreatitis and that these substances increase the global cytosolic $[Ca^{2+}]_i$ (Wang et al. 2009; Yang et al. 2009). A major part of this effect is due to inhibition of mitochondrial ATP production (Criddle et al. 2006). This was shown in patch clamp experiments in which the $[Ca^{2+}]_i$ rise normally evoked by a FAEE was abolished by supplying ATP directly to the cell interior via the patch clamp pipette (Criddle et al. 2006). In the absence of such an artificial ATP supply, the fatty acid–elicited reduction in the intracellular ATP level would prevent full Ca^{2+} pump function (both in the endoplasmic reticulum membrane and in the plasma membrane), limiting the capacity for getting rid of excess Ca^{2+} accumulated in the cytosol.

Ultimately, the steady-state $[Ca^{2+}]_i$ depends on the balance between Ca^{2+} entry and extrusion across the plasma membrane. Even a slight reduction in the function of the plasma membrane Ca^{2+} pump will cause a small rise in $[Ca^{2+}]_i$ as well as delaying significantly the process of restoring a near-normal Ca^{2+} level after a cytosolic Ca^{2+} signal (Ferdek et al. 2012). This will have functional consequences – increasing very significantly the risk of necrosis (Ferdek et al. 2012).

It is clear that the combination of ethanol and fatty acids would be particularly dangerous. As described above, ethanol is a potential releaser of intracellular Ca^{2+}, FAEEs are very potent Ca^{2+} releasers, and long chain fatty acids reduce mitochondrial ATP production, making it difficult for the cell to dispose of the Ca^{2+} released into the cytosol as both Ca^{2+} reuptake into the intracellular stores and Ca^{2+} extrusion via the plasma membrane require Ca^{2+}-ATPase function.

Although there is no doubt that the primary effect of FAEEs is to release Ca^{2+} from intracellular stores and that the release from the acid stores in the granular pole is particularly important for the intracellular trypsin activation, the reduction in the $[Ca^{2+}]_i$ in the endoplasmic reticulum is also very important because it is the reduction in the Ca^{2+} level in this organelle that activates Ca^{2+} entry channels in the plasma membrane that are known as store-operated or Ca^{2+}-release-activated Ca^{2+} channels (Parekh and Putney 2005). This mechanism has also been shown to operate in pancreatic acinar cells (Lur et al. 2009). It follows that it would in principle be possible to inhibit toxic Ca^{2+} signal generation initiating intracellular trypsinogen activation either by inhibiting the primary Ca^{2+} release inside the cells, for example, by CaM activation (Gerasimenko et al. 2011) or by inhibiting the store-operated Ca^{2+} channels. It has indeed been shown that the trypsinogen activation induced by stimulating the acinar cells with vastly excessive concentrations of cholecystokinin can be prevented by simply removing extracellular Ca^{2+} (Raraty et al. 2000).

9.2.1 Effects of Ethanol on Zymogen Granules

Chronic ethanol administration elevates the enzyme content of pancreatic ZGs (Apte et al. 1995), decreases the protease-inhibiting capacity of pancreatic acinar cells (Singh et al. 1982), and moreover inhibits exocytosis of ZGs via the apical membrane (Dolai et al. 2012). Ethanol was also shown to sensitize acinar cells for zymogen activation (Pandol et al. 1999; Gorelick 2003), to increase the fragility of pancreatic ZGs (Haber et al. 1993, 1994), and to stimulate exocytosis via the basolateral membrane (Cosen-Binker et al. 2007, 2008; Lam et al. 2007) initiating autodigestion of the pancreas (Apte et al. 1994, 2010). Recent studies on acinar cells also suggest that ethanol induces ER stress which is associated with accumulation of autophagic vacuoles (Lugea et al. 2010; Pandol et al. 2010). Both oxidative and non-oxidative metabolites of ethanol evoked similar effects on ZGs (Dolai et al. 2012). In summary, all of the above-mentioned effects of ethanol and ethanol products drive the pancreatic acinar cells to serious damage and cell death (Pandol et al. 2011).

10 Closing Remarks

The incidence of both acute and chronic pancreatitis is increasing worldwide (Hirota et al. 2012; Peery et al. 2012). In addition, AP is now the most frequent cause of hospitalization among the gastrointestinal diseases in the USA and its cost was estimated to be above 2.5 billion dollar per year (Peery et al. 2012). Besides the supportive care, there is still no specific pharmacological therapy against this inflammatory disease. In spite of significant recent progress in our understanding of the fundamental mechanisms by which pancreatitis is initiated, much remains to

be done. Although the mechanistic aspects of the toxic Ca^{2+} signal generation initiated by ethanol and ethanol products have become relatively clear, we still do not understand the crucial steps that link Ca^{2+} release from the ZGs with intracellular trypsinogen activation. Furthermore, one of the neglected areas of pancreatic research is the investigation of the undissociated acinar-ductal unit, which is, of course, the functional unit of the intact exocrine pancreas. Importantly, not only the physiological processes are jointly coordinated, but all pancreatitis-inducing factors damage both cell types and none of the cell type functions then remain appropriate. In this chapter, we have highlighted many physiological and pathophysiological processes, which need to be better understood in order to prevent and cure pancreatitis and thereby also reduce the incidence of pancreatic cancer.

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Chemosensory TRP Channels in the Respiratory Tract: Role in Toxic Lung Injury and Potential as "Sweet Spots" for Targeted Therapies

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Abstract Acute toxic lung injury by reactive inhalational compounds is an important and still unresolved medical problem. Hazardous gases or vapors, e. g. chlorine, phosgene, sulfur mustard or methyl isocyanate, are released during occupational accidents or combustion processes and also represent a potential threat in terroristic scenarios. According to their broad-range chemical reactivity, the mechanism of lung injury evoked by these agents has long been described as rather unspecific. Consequently, therapeutic options are still restricted to symptomatic treatment. However, in recent years, ion channels of the transient receptor potential (TRP) family have been identified to act as specific sensor molecules expressed in the respiratory tract and to engage defined signaling pathways upon inhalational exposure to toxic challenges. These pulmonary receptor molecules have been primarily characterized in sensory neurons of the lung. However, chemosensory molecules are also expressed in non-neuronal cells, e.g. in the lung epithelium as

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well as in the pulmonary vasculature. Thus, activation of respiratory chemosensors by toxic inhalants promotes a complex signaling network directly or indirectly regulating pulmonary blood flow, the integrity of the epithelial lining, and the mucociliary clearance of the bronchial system. This review gives a synopsis on reactive lung-toxic agents and their specific target molecules in the lung and summarizes the current knowledge about the pathophysiological role of chemosensory signaling in neuronal and non-neuronal cells in toxic lung injury. Finally, we describe possible future strategies for a causal, specifically tailored treatment option based on the mechanistic understanding of molecular events ensuing inhalation of lung-toxic agents.

Contents

1 Introduction

The respiratory system represents a delicate interface of our body with the environment providing a large surface area estimated to amount to about 100 m^2 depending on the method of determination (Colebatch and Ng 1992; Stone et al. 1992; Thurlbeck 1967). Since prehistoric times the respiratory tract of vertebrates is challenged with noxious inhalational substances, e.g. dust, fumes of wildfires, or ozone of natural origin. Thus, a multitude of defense and repair mechanisms have evolved protecting the respiratory system from harmful injury (Coleridge and Coleridge 1994; Comhair and Erzurum 2002). With the start of the industrial era a vast number of novel compounds occurred, representing potential toxic inhalation hazards (TIHs). Electrophilic or oxidizing substances like chlorine, phosgene, methyl isocyanate, nitrous gases, or nitrogen or sulfur mustards constitute one of the biggest groups of toxic inhalants (for review: Bessac and Jordt 2010).

There is a significant risk of exposure to TIHs, as most of these substances are produced by the chemical industry, either as main products, intermediates, or industrial pollutants. A famous sad example was the Bophal disaster: On December 3, 1984, more than 40 t of methyl isocyanate gas leaked from a pesticide plant in Bhopal, India, immediately killing at least 3,800 people and causing significant morbidity and premature death for many thousands more (Broughton 2005). Methyl isocyanate is an intermediate chemical in the production of carbamate pesticides. Other examples for omnipresent TIHs are chlorine and phosgene, two substances that are essential for the production of a wide range of industrial and consumer products. Additionally, chlorine is a common household chemical and a component of chlorine-based bleach. Bleach is regarded as one of the strongest airway irritants and has been discussed to play a causal role in the development of airway sensory hyper-reactivity (Quirce and Barranco 2010). Approximately 15 million tons of chlorine are produced annually in the United States alone to be used for water purification, pharmaceutical and disinfectant purposes (Samal et al. 2010).

Apart from the commercial and household use of potential lung damaging chemicals, a threat based on the misuse of lung toxicants cannot be ruled out. As most of the TIHs are widely accessible or are easy to synthesize, they can be considered as a terroristic threat. The use of chemicals, in first line TIHs, as warfare agents can be dated to World War I. On the 22nd of April 1915 German troops deployed chlorine at Ypres which is considered the starting point of chemical warfare in history. Thousands of tons of chemicals including chlorine, phosgene and sulfur mustard have been used by the war parties causing millions of casualties, specially due to lung injury (Kehe and Szinicz 2005). During World War II, large amounts of chemicals (e.g. nerve agents, blistering and lung agents) were produced and stockpiled. Blistering and lung agents were used especially by the Japanese Imperial Forces against Chinese troops and civilians and the remnants may still represent a health hazard (Hanaoka et al. 2006). In the European theater of war, no major operations with chemical weapons were carried out. So, most European casualties of chemical weapons during World War II resulted from accidental liberation of these agents, the most severe incident being the release of sulfur mustard from US freighter John Harvey after a bombing raid by the German air force (Alexander 1947). Of note, many thousands of tons of chemicals have been dumped in the Baltic Sea after World War II and still represent a potential environmental and health risk (HELCOM 1994; Sanderson et al. 2008; Sanderson et al. 2010).

Upon exposure to harmful inhalants, ancestral protective mechanisms mediate danger signals (burning sensations, lacrimation, blepharospasm, cough), induce detoxifying and antioxidant molecules (e.g. glutathione, urate, or metallothioneins), and activate self-cleaning mechanisms in the respiratory tract (cough, mucus production, elevated ciliary motility). These airway responses to TIHs are quite unspecific and uniform, in line with the wide array of potential inhalational threats, which display rather unrelated chemical structures. Thus, the molecular events involved in the recognition of harmful inhalational substances have been assumed to be equally unspecific. However, in recent years it has become clear that distinct

receptor molecules expressed on the cell membrane of respiratory cells are involved in the detection of TIHs. Especially, taste and olfactory receptors belonging to the superfamily of G protein coupled receptors and receptor-operated or chemosensory ion channels of the transient receptor potential (TRP) family have been identified in all parts of the airway system and found to play a pivotal role as defined target molecules of TIHs.

Similarly, the recent years have also seen a paradigm shift regarding the proposed mechanisms of acute lung injury evoked by reactive TIHs. Hitherto, the toxic action of TIHs has been mainly attributed to unspecific cell damage caused by reactions of the TIHs with biomolecules. In particular, oxidation of membrane lipids and alteration of DNA bases are scenarios which have been suggested to mediate the toxicity of TIHs. In line with this point of view, therapeutic interventions adopted so far are mainly symptomatic, i.e. application of steroids and β-adrenergic agonists as anti-inflammatory and broncho-spasmolytic agents, respectively, or administration of anti-oxidant molecules like e.g. N-acetyl cysteine (Bobb et al. 2005; de Lange and Meulenbelt 2011; Ghanei et al. 2008; Grainge and Rice 2010; Weinberger et al. 2011). Recently, the identification of molecular down-stream effectors of redox signaling in lung cells was a major step forward in the mechanistic understanding of the action of TIHs and possible pulmonary defense systems and will pave the way for more causal and specific therapeutic options. Especially, redox-regulated transcription factors like AP-1, Nrf2, and NF-κB have been identified as critical players activated upon exposure of airway cells to reactive inhalants and to control cell survival and inflammatory responses. Redox-regulated transcription can be orchestrated in a very complex manner by oxidation of cystein-residues. In the case of AP-1 and NF-κB signaling, oxidative stress in the cytosol can stimulate protein kinase-dependent cascades which ultimately lead to the activation of the transcription factors (Brigelius-Flohe and Flohe 2011). Furthermore, transcription factors like NF-κB and Nrf2 can be disinhibited via oxidative modification of inhibiting proteins directing the transcription factor towards ubiquitin-dependent proteasomal degradation under normoxic conditions (Muller and Hengstermann 2012). In contrast, oxidative stress in the nucleus can inhibit DNA binding of transcription factors, again via modification of critical cystein residues (Brigelius-Flohe and Flohe 2011).

Thus, substantial progress has been made in characterizing these events in the cytosol or in the nucleus directly controlling the redox-sensitive transcriptional regulation of target genes involved in the toxic effects of TIHs. The aim of this review is to point out the interconnections between membrane-signaling pathways engaged by defined chemosensory ion channels of the TRP family and the well-known down-stream effectors. Since different compartments of the respiratory tract are differentially impacted by TIHs, we assign the cellular responses in upper and lower compartments of the airways to the chemosensory structures expressed in these parts and the corresponding signaling pathways involved in the different pathophysiological events. Finally, we describe the perspective of a molecularly targeted therapy in respiratory toxicology based on selective agonists or antagonists of chemosensory molecules.

2 Cellular Events and Toxic Symptoms in Acute and Sub-acute Lung Injury by Reactive Inhalants

Each part of the airway system can be compromised by TIHs resulting in impaired lung function (e.g. toxic inhalation injury). Depending on the chemical properties (e.g. water solubility), chemical compounds affect different compartments of the lung. Particles >5 μm in diameter are filtered by impaction and are trapped in mucus coating the epithelium of the upper airways, thus not reaching deep lung compartments (Bonner 2008; Muhle and McClellan 2003). Particles of lower size, gases and aerosols are able to reach lower lung sections and finally the alveolar space. Deposition of TIHs depends mainly on water solubility (Fig. 1): Hydrophilic substances will dissolve in the mucous lining of the upper airways and will exert their toxic effects proximally (e.g. formaldehyde, ammonia, sulfur dioxide) (Bonner 2008; Muhle and McClellan 2003; Shusterman 1999; Tuorinsky and Sciuto 2008). In contrast, lipophilic substances with low water solubility (e.g. phosgene, ozone, oxides of nitrogen) will predominantly affect the lower respiratory tract, down to the alveolar space (Bonner 2008; Muhle and McClellan 2003; Shusterman 1999; Tuorinsky and Sciuto 2008).

Nevertheless, depending on the concentration of the TIH and time of exposure, chemicals can compromise the entire respiratory tract (Miller and Chang 2003; Tuorinsky and Sciuto 2008). As gases or vapors move by convection into the peripheral airways and then by diffusion to the alveolar-capillary membranes, TIHs reaching the alveolar space may have more profound health effects, because clearance due to gas exchange is much slower in this compartment (Urbanetti 1997). Pre-existing diseases of the airway system or damaged lung tissue due to former toxic inhalation injury may already compromise normal physiological lung function. In a mouse model of allergic asthma it was shown that additional inhalation of chemicals can enhance allergic airway inflammation in presensitized mice (Ban et al. 2006). Moreover, obstructive lung diseases (asthma, chronic bronchitis) can further constrain the elimination of TIHs leading to very severe health effects (Urbanetti 1997).

It is important to note that early clinical symptoms of airway irritation or damage do not always correlate with the clinical outcome or long-lasting health effects. Moreover, some TIHs may provoke serious health effects (e.g. pulmonary edema) after a latency period that may last several hours, during which signs and symptoms may not be evident, although cell damage has already occurred. Some chemicals have to undergo intracellular biotransformation processes before causing cell toxicity. It is remarkable that biotransformation is generally considered as a detoxification or defense process, but during this process, toxic metabolites may occur that can enhance lung injury (e.g. transformation of benzo(a)pyrene into reactive metabolites) (Cohen 1990; Smith and Brian 1991). If clinical symptoms arise instantaneously after exposure to certain TIHs, already existing target structures (e.g. cell-surface receptors) are affected and induce a cellular response. The biological effect depends on the activated sensor. In the case of stimulated nerve

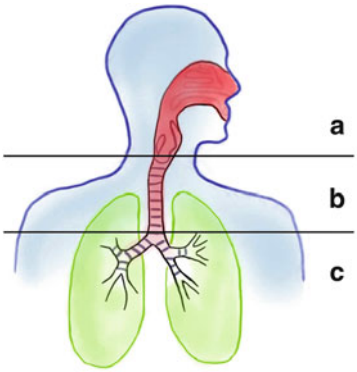

Fig. 1 Relationship between water solubility of TIHs and pulmonary target sites. Depending on the water solubility different primary target sites of TIHs in the human respiratory system can be determined. Highly water-soluble substances (e.g. ammonia, formaldehyde, sulfur dioxide) will affect mainly the upper respiratory system (*A*). Substances of intermediate solubility (e.g. ozone, chlorine) will affect tracheal and bronchial regions (*B*), whereas water insoluble substances (e.g. phosgene, oxides of nitrogens) will interfere with the lower respiratory system down to the alveolar spaces (*C*) (Bonner 2008, modified) (Shusterman 1999; Tuorinsky and Sciuto 2008)

endings, fast cellular responses are most likely and are a typical example for ion channel activation after a toxic stimulus inducing acute pain.

TIHs with similar chemical properties (e.g. chemical reactivity) are likely to interact with similar cellular targets. The cellular response and the resulting tissue lesions are based upon the molecular function of the affected cellular targets. Thus, TIHs with similar reactivity will provoke comparable or identical clinical symptoms, although their chemical structure may differ. Moreover, if specific cellular targets and the cellular response of certain TIHs are known, the pathophysiology of related substances can be predicted to a certain degree. Numerous studies have been conducted to assess the mode of action of toxic reactive inhalants. Unfortunately, the mode of action of most toxicants is largely unknown and the clinical picture of intoxication is generally nonspecific, which complicates diagnosis and therapy. Therefore, detailed knowledge about the molecular toxicology of lung irritants is required to develop specific therapeutic strategies.

The biological response of the respiratory system after exposure to TIHs is a result of complex interactions of multiple factors, including the intensity of exposure (concentration and time), condition of exposed tissues and also protective and regenerative mechanisms (Urbanetti 1997). Toxic effects can be mediated by direct interaction of the TIHs and respiratory cells leading to cellular dysfunction or cell death. Moreover, chemical substances may activate afferent nerve endings immediately provoking clinical symptoms. Inflammatory mediators, chemokines and interleukins released from affected lung epithelial cells, stimulated macrophages or other cell types will trigger additional biological effects such as cellular infiltration, vasodilation and leakage, pulmonary edema, mucus production, repair and remodeling processes.

3 Clinical Symptoms of TIH Exposure Related to the Nasopharynx

Usually the nasal mucosa, nasopharynx and larynx are exposed to the highest concentrations of toxicants as these structures represent the first line of defense after inhalation. Typical first symptoms will occur due to mucosal irritation like a tickle of the throat and cough which are both biological alert signs with the aim of initiating an escape reaction from the dangerous environment. Coughing and sneezing are triggered by activation of peripheral sensory nerve endings in the nasal airway lining. These nerve endings can be considered as a first defense mechanism against chemical challenges (Bessac and Jordt 2008, 2010). The purpose of this initial reflex-response is the removal of irritants from the nasal epithelial surface. After activation of nasal trigeminal, glossopharyngeal or vagal chemosensory neurons (type C and small diameter myelinated Aδ fibres) by TIHs, sensory-autonomic pathways elicit parasympathetic reflexes, mediated via cholinergic efferent neurons, resulting in secretions from nasal, lacrimatory and salivary glands in combination with the dilatation of vessels in the nasal mucosa and sinuses (Bessac and Jordt 2008, 2010). Mucosal swelling and leakage are common results leading to narrowing or obstruction of the nasal passage (Baraniuk and Kim 2007). The resulting edema of the larynx and glottis can be life-threatening due to acute obstruction of the upper airways.

4 Clinical Symptoms of TIH Exposure Related to the Tracheobronchial System

When irritants reach the tracheobronchial section, sensory neuronal activity can trigger tracheal and bronchial constriction via contraction of bronchial smooth muscle cells, mucus secretion, and neurogenic inflammation (Fig. 2), events which are considered as typical reactions after exposure to lung irritants (Miller and Chang 2003). Rhonchus is the typical diagnostic finding resulting from mucous secretion and constriction of the bronchial musculature. TIHs can be removed from the respiratory tract by different mechanisms. The airways of the lower respiratory tract are lined with cells that are predominantly ciliated or mucus producing. TIHs are trapped within the mucus layer and are removed by ciliated cells via mucociliary transport. Macrophages, either integrated in the epithelium or macrophages in the alveoli, are capable of phagocytosis and thereby clearing inhaled particles. Macrophages migrate to upper parts of the respiratory tract, where they are trapped in the mucociliar transport and are cleared from the airways. Moreover, activated macrophages release cytokines, chemokines, growth factors, proteolytic enzymes and ROS. These mediators recruit and activate other cells that either participate in tissue regeneration or may contribute to tissue remodeling processes. After exposure to lung irritants, mucociliary clearance may increase

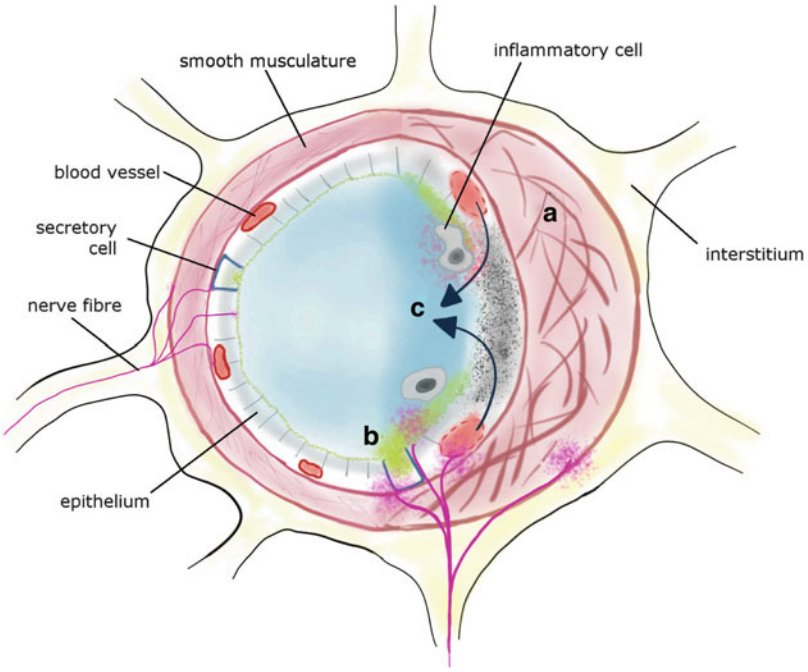

Fig. 2 Schematic section of a bronchus. Left part shows the physiological state, right part illustrates the (patho)physiological response after TIH exposure. Exposure with TIHs can lead to smooth muscle contraction (*A*) resulting in a reduction of airway diameter. Moreover, the epithelium lining the respiratory system and endothelium of pulmonary vessels can be affected, resulting in dysfunction, increased production of mucus (*B*) and fluid leakage into the aerial lumen (*C*), enhancing the reduction of airway diameter

when small or moderate concentrations of toxic chemicals are inhaled. However, high concentrations or very aggressive chemical substances may also interfere with the cilia of the respiratory epithelium or influence the epithelial integrity itself. As shown in an in vitro human bronchial co-culture cell culture model, sulfur mustard induced a widening of intercellular spaces and moreover, a loss in cell-matrix adhesion molecules was observed resulting in a decrease of transepithelial resistance (Pohl et al. 2009). Mucin production increased with resultant cessation of ciliar beat.

5 Clinical Symptoms of TIH Exposure Related to the Alveolar System

Chemical substances with little water solubility or high concentrations of TIHs are able to reach deep lung compartments, e.g. the alveoli. The complex damaging processes of TIHs in the alveolar space are depicted in Fig. 3 and include liberation

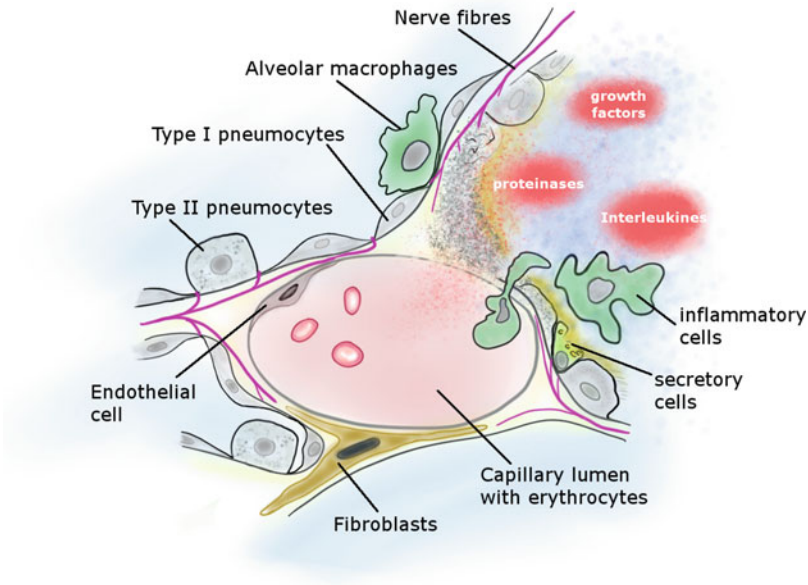

Fig. 3 Schematic illustration of the alveolar space and the cellular effects after TIH exposure. After exposure to TIHs, complex injury to lung tissue may occur. Damage to endothelial cells accompanied with epithelial damage, leads to alveolar edema and release of chemoattractants. Inflammatory cells (e.g. monocytes, neutrophils) migrate from the blood vessels into the alveolar space, secreting interleukins thus inducing and maintaining inflammation. Proteinases are activated, enhancing cellular damage. Mucin production by secretory cells is enhanced. Growth factors are secreted, inducing repair and/or remodeling processes

of inflammatory mediators, invasion of inflammatory cells, hypersecretion, and disturbance of cell-cell contacts leading to the destruction of the endothelial-epithelial barrier.

Apparently, type I alveolar epithelial cells (covering 90–95 % of the alveolar surface) appear to be most vulnerable to many lung toxicants probably because of their large surface area. Dysfunctional alveolar type I cells have to be replaced by new type I cells originating from type II alveolar cells. Alternatively, fibroblasts are activated to repair tissue lesions resulting in the formation of connective tissue and scars. Chemicals that affect both alveolar type I and type II cells have to be considered as extremely harmful, as regenerative cells are compromised and tissue damage cannot be adequately repaired. As the blood-air barrier is extremely thin (~0.4–1 μm), toxic substances may penetrate into lung capillaries (especially after damage to alveolar type I cells) and affect the endothelium, which is comparably vulnerable as epithelial cells type I. This damage will result in progressive impairment of the blood-air barrier and permeability will increase. Tight and adherence junctions between cells are loosened, facilitating loss of epithelial resistance. As a result, blood plasma enters from lung capillaries into the lung interstitium. The resulting interstitial edema increases the diffusion distance through the blood-air barrier leading to disturbance of gas exchange. First, edema

fluid is evacuated from the lung interstitium by the lymphatic system. Ongoing loss of epithelial and endothelial resistance will increase plasma leakage, overstraining the lymphatic system and resulting in alveolar edema. A pathological cycle is initiated: formation of edema foam, ascending from the alveolar space to the upper parts of the respiratory system, blocking bronchioles and impairing oxygenation. These events then cause increased pulmonary resistance which leads to further leakage into the alveoli. The whole process may result in a toxic lung edema that may be fatal.

Toxic lung edema due to increased alveolar permeability is hard to control, because tissue architecture is affected or even destroyed. Recovery of the integrity of the alveolar epithelial surface is an essential strategy after toxic inhalation injury. Especially, alveolar type I cells are vulnerable and may undergo cell death mediated either by direct TIH effects or indirectly by specific cytokines released from affected cells, residual structural cells or activated macrophages. Various acute inflammatory mediators are expressed in the lung during acute lung injury (e.g. IL-1α, IL-8, TNF-α, C3b, leukotrienes). In parallel, proteases are released from infiltrating neutrophils and mononuclear cells and degrade the extracellular matrix. Resulting debris is subsequently cleared by macrophages (attracted and differentiated from bone marrow and circulatory pools). Next, growth factors (e.g. TGF-ß, CTGF, PDGF) stimulate basal cells (type II alveolar cells) to differentiate into type I cells and other cell types reconstitute connective tissue, vascular tissue, and nerve endings. A careful balance of catalytic and anabolic processes is mandatory for a complete physiological repair. If one of the processes is gaining an upper hand, fibrosis due to enhanced production of collagen and connective tissue or emphysema due to enhanced protease activity may occur (Bonner 2008).

6 Members of the TRP Channel Family as Chemosensory Molecules in the Airways

Virtually all pathophysiological processes mentioned above are regulated or modulated by activation of chemosensory molecules in the plasma membrane of cells in the respiratory system. In particular chemosensory TRP channels play a pivotal role in the detection of TIHs, in the control of adaptive responses, and in the initiation of detrimental signaling cascades.

TRP channels represent one of the largest superfamilies of ion channels in the human genome (Clapham et al. 2001; Montell et al. 2002; Nilius and Owsianik 2011; Pedersen et al. 2005). Mammalian TRP channels can be subdivided into six families, i.e. TRPC, TRPM, TRPV, TRPP, TRPML, and TRPA channels as shown in Fig. 4. The common structural features of the members of the TRP superfamily are six transmembrane-spanning domains (TM1-6) and a putative pore region (P) located between transmembrane domains 5 and 6 (see Fig. 5). TRP channels are permeable to cations with striking differences in ion selectivity. Many TRP

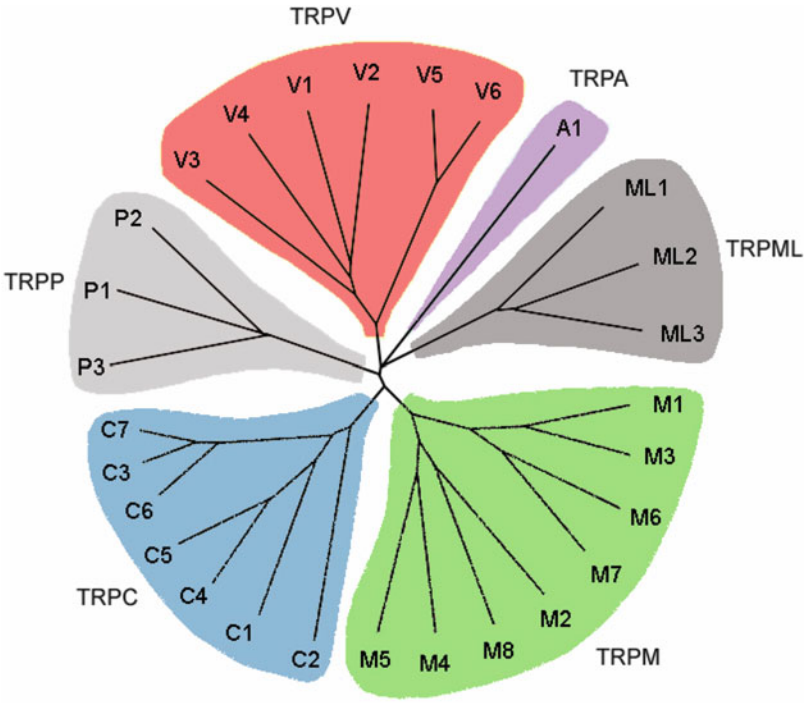

Fig. 4 Phylogenetic tree of TRP channels. The relationships between the TRP channels are illustrated by the branch lengths

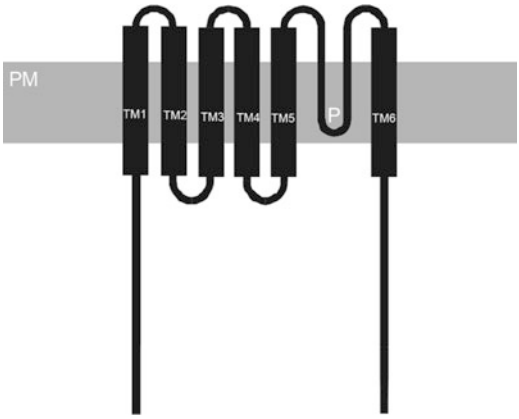

Fig. 5 Common structural features of TRP channels. TRP channels have six transmembrane regions connected by intra- and extracellular loops. A loop between transmembrane domain 5 and 6 is essential for the formation of a pore in a functional TRP channel tetramer at the plasma membrane. *PM* plasma membrane, *TM1-6* transmembrane-spanning domains 1–6, *P* pore region

channels conduct monovalent cations like Na^+ or K^+ as well as bivalents like Ca^{2+}, Mg^{2+} or bivalent cations of transition metals. Some TRP channels like TRPM4 and TRPM5 show high selectivity for monovalent cations, other channels like TRPV5 or TRPV6 are highly selective for Ca^{2+} (for review: Owsianik et al. 2006).

The archetypal TRP channel, eponymous for the whole super-family, is the product of the *trp* gene of the fruit fly, *Drosophila melanogaster*. Cloning of the *Drosophila* TRP protein was preceded by the characterization of a mutant fruit fly with a visual defect. In this mutant strain, termed *transient receptor potential* (*TRP*) (Minke 1977; Montell et al. 1985), exposure to light only led to a short-lived depolarizing current due to an inactivating mutation of the TRP channel, which is an indispensable component of the phototransduction cascade in *Drosophila* (Hardie and Minke 1992; Montell et al. 1985; Montell and Rubin 1989). In some analogy to *Drosophila* TRP, many mammalian TRP channels exert sensory functions, although mostly outside of the visual system. Thus, TRP channels like TRPV1 (Caterina et al. 1999; Cesare et al. 1999; Tominaga et al. 1998), TRPV2 (Caterina et al. 1999), TRPV3 (Peier et al. 2002b; Smith et al. 2002; Xu et al. 2002), and TRPV4 (Guler et al. 2002; Watanabe et al. 2002) were reported to be activated by elevated temperature and are involved in thermo-sensing (for review: Tominaga 2007), whereas TRPM8 is activated by cold temperatures (McKemy et al. 2002; Peier et al. 2002a). Other TRP channels like TRPM5 are part of the signal transduction pathway engaged by taste receptors (Perez et al. 2002; Zhang et al. 2003). Of note, TRPM5 channel activity also depends on temperature (Talavera et al. 2005), a fact that explains why sweet or bitter food has a more intense taste in higher temperatures. Furthermore, TRP channels like TRPV4 react to mechanical stimuli in various cell types and organs (for review: Liedtke 2007; Plant and Strotmann 2007; Yin and Kuebler 2010) including the lung vasculature (Jian et al. 2008).

The most important function of TRP channels in the context of this review is their role as chemosensory molecules. In the respiratory system, especially TRPV1, TRPV4, TRPA1, and TRPM8 are involved in the detection of potentially harmful inhalants. However, these channels display a remarkable promiscuity with regard to their activators. For example, TRPA1 is activated by a large number of chemically unrelated substances (Table 1). Furthermore, TRP channels of the TRPC family are expressed in the lung and form part of redox-regulated signaling pathways. The activation of these TRP channels by TIHs results either from covalent modification of cystein residues of the ion channel or from phospholipase C (PLC)-regulated changes in lipid messengers (diacylglyercol, phosphatidyl-inositolphosphate) or intracellular calcium levels. In the first scenario, the TRP channel can be activated directly by reaction with an electrophilic TIH (Brone et al. 2008; Hinman et al. 2006; Macpherson et al. 2007a) or the reaction of the TIH with constituents of the plasma membrane leading to the generation of secondary TRP activators (Taylor-Clark et al. 2009; Trevisani et al. 2007). Regarding PLC-promoted signaling events leading to the activation of TRP channels, either stimulation of $G_{q/11}$-coupled protease-activated receptors (PARs) or purinergic receptors with subsequent activation of PLC-β or stimulation of redox-sensitive PLC isoforms is involved (see below).

Of note, some pulmonary TRP channels are involved in O_2 sensing by detecting hypoxic as well as hyperoxic states (for review: Takahashi et al. 2012). Hypoxia can lead to an activation of TRPA1- or TRPC6-promoted calcium responses

Table 1 Examples of different TRPA1 activators with EC_{50} values (for human TRPA1) and putative activation mechanism.

Substance	EC_{50}	Putative mechanism	Literature
2-Pentenal	?	Covalent cysteine modification	Bautista et al. (2006)
Acetaldehyde	77 μM	Covalent cysteine modification	Bang et al. (2007)
Acrolein	1-5 μM	Covalent cysteine modification	Andre et al. (2008), Bautista et al. (2006)
Allyl isothiocyanate (AITC)	2–11 μM	Covalent cysteine modification	Bautista et al. (2005), Hinman et al. (2006), Jordt et al. (2004), McNamara et al. (2007)
Chlorobenzylidene malononitrile (CS, tear gas)	0.2 μM	Covalent cysteine modification	Brone et al. (2008)
Crotonaldehyde	16 μM–5.1 mM	Covalent cysteine modification	Andre et al. (2008), Mukhopadhyay et al. (2011)
Formaldehyde	200–357 μM	Covalent cysteine modification	Macpherson et al. (2007b), McNamara et al. (2007)
Methylisocyanate	25 μM	Covalent cysteine modification	Bessac et al. (2009)
Ammonium chloride	9.2 mM	pH shift	Dhaka et al. (2009), Fujita et al. (2008)
Nicotine	>10 μM	Non-covalent	Talavera et al. (2009)
Isoflurane	180 μM	?	Matta et al. (2008)
Hydrogen peroxide	23–297 μM	Oxidation of cystein residues	Andersson et al. (2008), Hill and Schaefer (2009), Sawada et al. (2008), Takahashi et al. (2008a)
Hypochlorite anion	Equivalent to 7–11 ppm of chlorine	Oxidation of cystein residues	Bessac et al. (2008)

(Fuchs et al. 2011; Takahashi et al. 2011; Weissmann et al. 2006). TRPA1 acting as an O_2 sensor is expressed in vagal neurons in the lung and its activation by hypoxia occurs via reduced activity of proline hydroxylases (PHDs). In normoxia PHDs oxidize a proline residue in TRPA1 (Pro394) thus inhibiting the channel (Takahashi et al. 2011). Hyperoxic states lead to the direct oxidation of cystein residues of TRPA1 (Cys633 and Cys856), a modification, which overrides the inhibition of TRPA1 by proline hydroxylation (Takahashi et al. 2011). Whereas TRPA1 is directly modified by the ambient O_2 concentration, TRPC6 is activated by hypoxic states via the enhanced generation of reactive oxygen species during hypoxia, which in turn lead to an accumulation of diacyl glycerol (DAG) (Weissmann et al. 2006). Thus, TIHs leading to obstruction of the airways via bronchoconstriction or increased mucus secretion can indirectly activate TRPA1 or TRC6. In contrast, oxidizing TIHs could potentially mimick hyperoxic conditions and by this means directly activate TRPA1.

To explain the role of TRP channels in pulmonary responses upon exposure to TIHs in more detail, this review will first focus on the involvement of TRP channels in irritative responses of the upper respiratory tract. Afterwards, we will discuss the special role of TRPA1 and TRPV1 in the cough reflex. Thereafter, the impact of TRP channels, especially TRPV4, in the regulation of mucociliary clearance will be addressed. Finally, the role of members of the canonical family of TRP channels, TRPCs, in redox-regulated pathways will be discussed.

7 TRP Channels and Irritative Responses in the Upper Respiratory Tract

As stated above, the mucosa of the eyes and the mucosa of the nasopharynx are first-line target of TIHs, especially those with high water solubility. Of note, all features of mucosal responses after exposure to TIHs, i.e. lacrimation, sternutation, nasal discharge, and irritative pain, are regulated by TRP channels.

7.1 Irritant-Induced Lacrimation

With regard to lacrimation responses several TRP channels are involved. TRPM8, TRPA1, and TRPV1 are known to be functionally expressed in nerve terminals in the cornea (Murata and Masuko 2006) and prototypical TRP agonists like menthol (TRPM8), allyl isothiocyanate (TRPA1), and capsaicin (TRPV1) lead to increased lacrimation (Parra et al. 2010). Of note, TRPM8, which is activated by cool temperatures, appears not to be involved in lacrimation caused by irritants, but to regulate basal tearing rate via a cooling effect of the evaporating tear film (Parra et al. 2010). Consequently, low concentrations of the TRPM8 agonist menthol led to increased tear flow in mice without inducing nocifensive effects, whereas high concentrations of menthol induced nocifensive behavior in a TRPM8-independent manner (Robbins et al. 2012). Apart from the aforementioned TRP channels expressed in corneal nerve endings, other thermo- and osmosensitive TRPV channels, i.e. TRPV2, TRPV3, and TRPV4 have also been described in corneal epithelial cells (Mergler et al. 2011). However, their (patho)physiological functions in the eye still await further clarification. Of note, a non-TRPM8 and non-TRPA1 component is involved in tear flow excited by drying of the cornea, which leads to increased osmolarity of the tear fluid (Hirata and Oshinsky 2012). Thus, especially the osmosensitive TRPV4 channel could be involved in the regulation of basal tear flow. Regarding the role TRP channels in irritant-induced lacrimation, TRPV1 and TRPA1 play the predominant role. Thus, well known lachrymators like constituents of tear gas (e.g. 2-chlorobenzalmalononitrile, CS), pepper spray (capsaicin) or

onions (allicin) exert their irritative effects via activation of TRPA1 or TRPV1 (Salazar et al. 2008).

7.2 Role of TRPA1 and TRPV1 in Nasal Irritation by TIHs

Irritants acting on the nasal mucosa lead to a stimulation of trigeminal C fiber nerve endings with subsequent irritative responses. Many well-known trigger substances of these trigeminal reflexes, like capsaicin, menthol, or mustard oil directly target TRP channels. Capsaicin, for instance, has been extensively characterized as a tool to investigate airway reflexes mediated via C fibers (Coleridge and Coleridge 1977; Coleridge et al. 1965; Fuller et al. 1985). Application of capsaicin or other TRPV1 agonists lead to the release of substance P or CGRP by nerve endings in the nasal mucosa (Lundberg et al. 1984; Lundblad et al. 1983; Malis et al. 2001; Petersson et al. 1989) and thus, contributes to neurogenic inflammation in the nasopharynx. Functional studies have suggested that TRPV1, TRPA1, and TRPM8 are expressed in trigeminal neurons innervating the nasal cavity (Damann et al. 2006; Frasnelli et al. 2011; Karashima et al. 2007). Thus, these chemosensory channels have been postulated as critical sensor molecules in the nasopharynx involved in the detection of oxidizing or electrophilic TIHs (Bessac et al. 2008; Gerhold and Bautista 2009).

Interestingly, TRPV1 expression in the nasal mucosa has been also demonstrated in non-neuronal cells, e.g. in epithelial cells, vascular endothelium, and submucosal glands (Seki et al. 2006). Furthermore, stimulation of human cells from nasal or bronchial epithelium with capsaicin led to the production of interleukin-6 (IL-6) which could be blocked by co-treatment with TRPV1 antagonist capsazepine (Seki et al. 2007).

7.3 Cough Reflex: Role of TRPA1 and TRPV1

The cough reflex is an important danger signal upon exposure to harmful inhalants and protects the airways by removing ingested stimuli. However, chronic cough severely impacts the quality of life and represents an important, but often intractable reason for patients to consult a physician. Therefore, novel therapeutic options to treat chronic cough are urgently needed. In this context, it is important to note that cough can be induced via two distinct mechanisms, i.e., either by chemical irritation stimulating a reflex mediated via C fibers or by mechanical stimuli, which produce a cough reflex via Aδ fibers (for review: Brooks 2011). Whereas the role of TRP channels in mechanical induced cough is still not clearly defined, the role of these channels in chemically-induced cough is well established. Especially, TRPA1 and TRPV1 channels represent pivotal factors in regulating irritant-induced cough (for review: Bessac and Jordt 2008; Geppetti et al. 2010; Materazzi et al. 2009) and are promising therapeutic targets (for review: Banner et al. 2011; Preti et al. 2012).

Thus, TRPA1 agonists like allyl isothiocyanate (AITC) and TRPV1 agonists like capsaicin are potent inducers of cough reflex. Vice versa, TRPA1- and TRPV1-inhibitors have been shown to block cough responses provoked by exposure to the respective agonist in animal models (Brozmanova et al. 2012; Grace et al. 2012). In a comparative study using the guinea pig model, the TRPV1 activator capsaicin was more efficient to induce cough than the TRPA1 activator AITC (Brozmanova et al. 2012). Regarding potential endogenous activators of the TRP-promoted cough reflex, it has been shown that prostaglandin E2 (PGE2) and bradykinin (Bk) can induce a tussive response in animal models and are able to activate TRPA1 and TRPV1 in human, mouse and guinea pig airway cells (Grace et al. 2012). These findings are commensurate with previous results showing that Bk can activate TRPA1 via a PLC- and cAMP-dependent protein kinase (PKA)-dependent mechanism (Bandell et al. 2004; Wang et al. 2008). Interestingly, the contribution of these channel in Bk- or PGE2-promoted cough was additive: thus, the sole use of TRPV1- and TRPA1-blockers led to partial inhibition of the tussive response to both stimuli, whereas combination of TRPV1 and TRPA1 inhibitors completely abrogated cough responses (Grace et al. 2012). While the exact activation mechanism of TRPA1 and TRPV1 by PGE2 and Bk is not fully elucidated, these findings indicate that a number of exogenous or endogenous processes leading to the release of inflammatory mediators including Bk and PGE2 could potentially lead to an activation of these cough-associated TRP channels.

The importance of human TRPs in the pathogenesis of cough has been recently investigated in a genetic analysis of single nucleotide polymorphisms (SNPs) of TRPV1 and their relationship with cough symptoms in 844 asthmatic and 2,046 non-asthmatic patients (Smit et al. 2012). Of note, seven SNPs of TRPV1 showed a statistically significant association with cough in non-asthmatics (six SNPs with nocturnal cough and one with usual cough). Furthermore, four SNPs of TRPV1 were associated with an increased risk of cough in persons (asthmatics and non-asthmatics) with exposure to irritants (Jugg et al. 2011). For other TRP channels like TRPV4 and TRPA1 no significant relationship of SNPs with cough could be detected in this study.

These findings suggest that TRPV1 plays a crucial role in mediating chemically-induced cough-responses. However, with regard to reactive, electrophilic inhalants like acrolein, croton aldehyde, methyl isocyanate, or AITC, TRPA1 may be more important as primary target. As stated above, electrophilic and oxidizing compounds are able to activate TRPA1 as well as TRPV1 via covalent modification of cystein residues (Hinman et al. 2006; Macpherson et al. 2007a; Salazar et al. 2008). However, the electrophilic α, β-unsaturated aldehyde acrolein activated C fibers in wild-type and TRPV1-knockout mice, a response that could not be modified by additional treatment with TRPV1 blocker iodoresiniferatoxin indicating that at least in this murine model TRPV1 is not the primary target of a prototypical electrophilic inhalant (Symanowicz et al. 2004). The importance of TRPA1 in response to electrophilic inhalants is further supported by findings in guinea pigs, which presented pronounced coughing upon challenge with AITC, cinnamaldehyde, acrolein, or croton aldehyde, which could be blocked by

inhibition of TRPA1 using the selective antagonist HC-030031 but not by inhibition of TRPV1 using the selective blocker capsazepine (Andre et al. 2009).

7.4 TRPV4 and Mucociliary Clearance

As mentioned before, a critical function of the bronchial system involved in adaptive responses to exposure to TIHs is related to the mucociliary clearance system. The driving force for increased ciliary activity in the bronchial system is an increase of the intracellular calcium concentration in ciliary cells (Salathe and Bookman 1995). Therefore, chemosensory, calcium-permeable channels of the TRP family are plausible candidates to modulate ciliary beat frequency after chemical irritation. In fact, TRPV4 expression has been described in many ciliary cells, e.g. in the ovarian duct (Andrade et al. 2005), in cholangiocytes (Gradilone et al. 2007), and also in bronchial epithelial cells (Lorenzo et al. 2008; Seminario-Vidal et al. 2011).

In ciliated cells of the oviduct treatment with high viscosity fluid increased ciliar activity, an effect that could be blocked by loading cells with TRPV4 antibodies and was mimicked by TRPV4 agonists (Andrade et al. 2005) indicating that TRPV4 is a major component of mechanically induced ciliary activity in these cells. In contrast, TRPV4 appeared not to be critically involved in ciliary activity upon viscous challenge in bronchial epithelium, since highly viscous fluid did not provoke different effects in tracheal epithelial cells from wild-type or TRPV4-knock-out mice (Lorenzo et al. 2008). However, stimulation of tracheal cells with ATP, a well-known activator of ciliary beat frequency in the bronchial system, led to a receptor-operated calcium signal strongly dependent on TRPV4 (Lorenzo et al. 2008). Intriguingly, activation of TRPV4 has been described to stimulate the release of ATP by airway epithelial cells induced by cell swelling in a pathway involving pannexin 1, RhoA and myosin light chain phosphorylation (Seminario-Vidal et al. 2011).

These findings suggest that TRPV4 and ATP may form part of a self-amplifying system with TRPV4 responsible for ATP-promoted calcium signaling and subsequently triggering ATP release after activation (Fig. 6). Moreover, ATP release by airway cells has been demonstrated after treatment with the lung toxicant bleomycin and has been proposed to form a universal danger signal (Riteau et al. 2010).

8 Pulmonary TRPC Channels

In recent years evidence for an involvement of TRPC channels in lung function and toxicity became evident. Although TRPC channels are not the primary sensor molecules in different lung tissues, they are essential members of signal

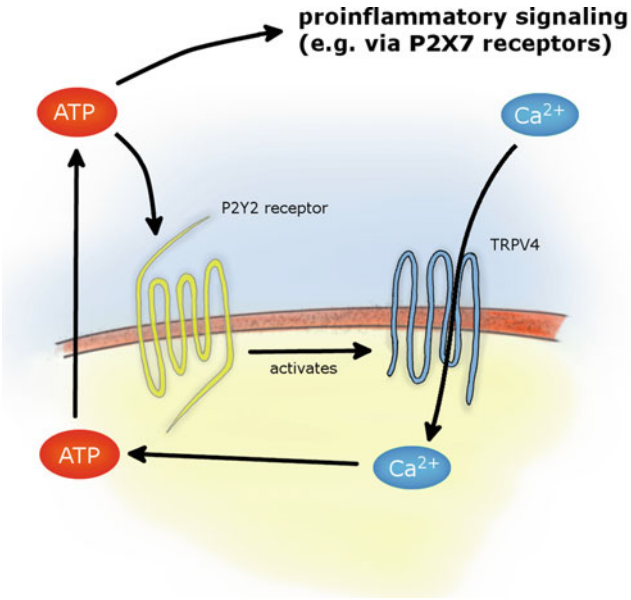

Fig. 6 Involvement of TRPV4 in ATP-signaling in bronchial epithelium. Stimulation of metabotropic purinoceptors (e.g. P2Y2) leads to the activation of TRPV4 which in turn can trigger the release of ATP. By this means, the interplay of ATP-dependent G protein coupled receptors and TRPV4 may sustain a self-amplifying signaling cascade with high extracellular levels of ATP representing a danger signal in lung epithelial cells

transduction cascades mediating toxicity in the targeted cells. TRPC channel activity is induced after ligand binding to a receptor (e.g. a $G_{q/11}$ protein-coupled receptor) and complex intracellular signaling cascades (Dietrich et al. 2005).

The seven TRPC family members can be grouped into subfamilies on the basis of their amino acid sequence similarity. While TRPC1 and TRPC2 are almost unique, TRPC4 and TRPC5 share ~65 % identity. TRPC3, 6 and 7 form a structural and functional subfamily with 70–80 % identity at the amino acid level and their ability to be activated by DAG (Dietrich et al. 2005; Hofmann et al. 1999; Okada et al. 1999), a product of phosphatidylinositol 4,5-bisphosphate cleavage by activated phospholipases C. In all eukaryotic cells, activation of phospholipase C (PLC)-coupled membrane receptors by hormones or neurotransmitters leads to an increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. Both activation of PLCβ and ε-isozymes by G protein coupled receptors and of PLCγ isoforms by receptor tyrosine kinases results in the hydrolysis of phosphatidyinositol 4,5-bisphosphate and the generation of inositol 1,4,5-trisphosphate (IP_3) and DAG. While it seems clear that TRPC3/6/7 channels are directly activated by DAG and are responsible for **r**eceptor-**o**perated **c**alcium **e**ntry (ROCE) (Hofmann et al. 1999; Okada et al. 1999), the regulation of other members and their involvement in **s**tore **o**perated **c**alcium **e**ntry (SOCE) is discussed controversially. SOCE occurs when inositol 1,4,5-trisphosphate (IP_3) or some other signal discharges Ca^{2+} from intracellular

stores in the endoplasmic reticulum (ER). The subsequent fall in the ER Ca^{2+} concentration then signals to the plasma membrane and activates store-operated channels. A major breakthrough in understanding SOCE was the identification of the calcium sensor STIM (Liou et al. 2005; Roos et al. 2005; Zhang et al. 2005) and the Orai channels (Cahalan 2009; Feske et al. 2006; Vig et al. 2006) in 2005 and 2006, respectively. STIM 1 which is predominantly located in the ER, is a single transmembrane domain protein containing two N-terminal Ca^{2+}-binding EF hands in the ER lumen and is therefore ideally situated to detect calcium levels in the ER. Upon Ca^{2+} release reduced calcium levels induce STIM redistribution to punctae and opening of store-operated channels in the plasma membrane. The so called Orai proteins with only four predicted transmembrane domains and intracellular C- and N-termini, were identified as the pore forming unit of store-operated channels by the analysis of T cells from patients with severe combined immunodeficiency (SCID) reviewed in Cahalan (2009). Although Orai 1 channels can multimerize with themselves to form pore forming units, it was also suggested that Orai molecules might interact with TRPC channels heterologously expressed in HEK293 cells to form store-operated channels (Liao et al. 2009). Moreover, evidence supporting the interaction of the calcium sensor STIM1 with TRPC proteins was presented for the same heterologous expression system (Yuan et al. 2009a, b).

TRPCs are also regulated by phosphorylation. TRPC3 channels can be inactivated by direct phosphorylation of amino acids threonine 11 (Thr 11) and serine 263 (Ser 263) by protein kinase G (PKG) (Kwan et al. 2004) and by protein kinase C (PKC)-mediated phosphorylation of Ser 712 (Trebak et al. 2005). The receptor tyrosine kinase Src phosphorylates TRPC3 at Tyrosine 226 (Tyr 226) and inhibits TRPC3 activity (Kawasaki et al. 2006), while Fyn another member of the Src family increases TRPC6 activity (Hisatsune et al. 2004). It has been shown that TRPC6 is negatively regulated after phosphorylation of Thr 69 by PKG (Takahashi et al. 2008b). Most interestingly, this phosphorylation is essential for anti-hypertrophic effects of phosphodiesterase 5 inhibitors (Kinoshita et al. 2010; Koitabashi et al. 2010; Nishida et al. 2010), which are also important therapeutics for pulmonary hypertension (Reichenberger et al. 2007).

TRPC channels can form functional homo- and heterotetramers within two defined subgroups (TRPC1/4/5 and TRPC3/6/7) excluding TRPC2 which is a non-functional pseudogene in humans (Goel et al. 2002; Hofmann et al. 2002). Novel combinations of TRPC1- with TRPC4 or 5 together with TRPC3, 6 or 7 in tetrameric complexes with three different TRPC family members have also been identified in HEK293 cells, as well as in embryonic brain, but not in adult rat tissues (Strubing et al. 2003). Evidence for the importance of the highly conserved pore region in the TRPC tetramer is derived from site-directed mutagenesis which results not only in the complete loss of channel activity upon heterologous expression, but also in a dominant-negative effect of a mutated channel monomer on functional homo- or heteromeric channel tetramers (Hofmann et al. 2002).

The TRPC family includes seven members (TRPC1-7), but only six are functional in humans, because TRPC2 is a pseudogene in the human genome. TRPC7

expression in lung is sparse and is predominantly identified in alveolar macrophages (Finney-Hayward et al. 2010). Expression of the other TRPC channels in pulmonary tissues and recent evidence concerning their potential function is summarized in the next paragraph.

9 Role of TRPC1, TRPC3, TRPC4, and TRPC5 in the Lung

Although **TRPC1** was the first mammalian TRP channel to be cloned, its physiological functions are still elusive. It is not even clear, if TRPC1 is an ion channel or merely an adaptor protein regulating ion channel activity of other TRPC members (Storch et al. 2012). Homomeric TRPC1 complexes do not translocate to the plasma membrane (Hofmann et al. 2002), but TRPC1 as a member of heteromeric TRPC1/5 or TRPC1/4 complexes has a high impact on TRPC4/5 currents (Strubing et al. 2001) and decreases calcium permeability in such channel complexes (Storch et al. 2012). It has been reported that TRPC1 is important for the proliferation of bronchial smooth muscle cells, which may induce thickening of the airways facilitating airway hyperreactivity (summarized in Li et al. 2003). Along these lines, TRPC1 is up-regulated in proliferating airway smooth muscle cells while TRPC1 down-regulation inhibits cell proliferation (Sweeney et al. 2002a, b). However, it is not clear, if proliferation is influenced exclusively by TRPC1 channels or by TRPC1/4 channel complexes, because heteromeric TRPC1/4 channel complexes seem to be more important for the lung extra-alveolar endothelial barrier than for the capillary endothelial barrier (summarized in Cioffi et al. 2009). **TRPC3** expression is up-regulated by tumour necrosis factor (TNF)-α in human airway smooth muscle cells (White et al. 2006). TNF-α is a pro-inflammatory cytokine which has been implicated in asthma and COPD because it increases airway hyperresponsiveness (AHR) by increasing airway smooth muscle Ca^{2+}. Therefore, TNF-α blockage might represent a promising pharmacological target for the treatment of asthma and COPD (summarized in Amrani 2007). The function of **TRPC4** in lung vascular endothelial cells was analysed using TRPC4−/− mice and wild-type controls. A defect in thrombin-induced Ca^{2+} influx is associated with reduced actin stress fiber formation and reduced endothelial cell retraction response. In isolated perfused lungs thrombin receptor activation increased the microvessel filtration coefficient (Kfc) by 2.8-fold in wild-type lungs but only 1.4 in TRPC4−/− lungs. This data provides evidence for a role of TRPC4 together with other ion channels in lung endothelial permeability (Tiruppathi et al. 2002). Recently **TRPC5** was detected in the apical membrane of neuroepithelial bodies in the intrapulmonary airway epithelium by TRPC5 specific antibodies. However, it remains elusive if and how TRPC5 is involved in signal transduction cascades of these cells (Lembrechts et al. 2012).

10 TRPC6: Involvement in Redox-Sensitive Pathways in the Lung

Many studies were already published on the role of **TRPC6** channels in the airways. Indeed, TRPC6 function in the lung might be particularly important, because it is the most prominently expressed TRPC channel in lungs (Hofmann et al. 2000). Therefore, we set out to analyze allergic responses in a TRP6-deficient mouse model expecting a reduced response in comparison to wild-type (WT) mice. However, we found an increased methacholine-induced AHR in TRPC6-deficient mice compared to WT mice. This surprising finding is most probably due to compensatory up-regulation of TRPC3 in airway smooth muscle cells (Sel et al. 2008). By experimental inflammation induced by intraperitoneal ovalbumin (OVA) sensitization followed by OVA aerosol challenges we were able to detect a decreased level of T-helper type 2 (Th2) cytokines (IL-5 and IL-13) as well as reduced IgE levels in the blood of TRPC6−/− mice compared to WT mice. Mucus production in goblets cells from challenged mice however was not altered in TRPC6-deficient mice (Sel et al. 2008). This data points to an important role of TRPC6 in the immune responsive rather than in airway smooth muscle tissues. Because neutrophil numbers are increased in the sputum of COPD and asthma patients, TRPC6 function in these cells might be also important for the progress of the diseases. Migration of TRPC6−/− neutrophils in response to macrophage inflammatory protein-2 (MIP2 also known as CXCL2) was reduced compared to WT neutrophils (Damann et al. 2009). In the same report an involvement of TRPC6 in cytoskeletal rearrangements during neutrophil migration was demonstrated suggesting an important role of TRPC6 in the migration of lung neutrophils. TRPC6 was also identified in other immune cells of the lung like alveolar macrophages (Finney-Hayward et al. 2010). Most interestingly, TRPC6 mRNA expression was significantly increased in macrophages obtained from COPD patients compared to healthy controls, while TRPC3 and TRPC7 levels remained unchanged (Finney-Hayward et al. 2010).

Under conditions of alveolar hypoxia induced by obstruction of the lung by particulate matter the pulmonary arterial vessels constrict diverting blood flow to the well ventilated areas in the lung to ensure maximal oxygenation of the venous blood by adequately matching ventilation with perfusion. This alveolar hypoxia-mediated vasoconstrictive phenomenon is known as acute hypoxic pulmonary vasoconstriction (HPV) and was first described by von Euler and Liljestrand in 1946. Sustained pulmonary vasoconstriction (chronic HPV) is often accompanied by vascular remodelling, i.e. the muscularization of smaller arteries and arterioles due to SMC proliferation and migration. In severe forms of pulmonary hypertension (such as idiopathic and familial PAH), pulmonary artery remodelling is extensive resulting from intimal fibrosis and medial hypertrophy. Such occlusion of the pulmonary arterioles is associated with the formation of plexiform lesions resulting from the proliferation of endothelial cells, migration and proliferation of SMC, and accumulation of circulating cells (including macrophages and

endothelial progenitor cells). In both pulmonary and systemic circulation, the blood flow and intraluminal pressure are mainly regulated by changes in vessel diameters.

TRPC6 was found to be up-regulated in hypoxia-induced, as well as idiopathic chronic pulmonary hypertension (IPAH) (Lin et al. 2004; Yu et al. 2004). IPAH is caused by excessive smooth muscle proliferation and results in right heart failure. IPAH is associated with up-regulated expression of TRPC6 and TRPC3, but not TRPC1 genes at the mRNA and protein levels (Kunichika et al. 2004; Yu et al. 2004). Most interestingly, in patients with IPAH the allele frequency of a C/G single nucleotide polymorphism (SNP) at position −254 upstream of the transcription start of the TRPC6 gene is significantly higher (12 %) than in normal subjects (6 %). This polymorphism creates a binding site for nuclear factor-κB (Yu et al. 2009). Therefore, TRPC6 might also be induced at least in patients with the C/G polymorphism during inflammation caused by toxic irritants in the lung.

During an extensive analysis of TRPC6−/− mice, we set out to determine pulmonary arterial pressure (PAP) in isolated lung during acute (<20 min) and prolonged (60–160 min) hypoxia. Much to our surprise, acute HPV was completely absent in TRPC6−/− mice, while vasoconstriction after prolonged hypoxia was not significantly different in TRPC6−/− mice compared to wild type mice. These data show for the first time, that the acute hypoxic vasoconstrictor response and the prolonged phase are regulated by different molecular mechanisms. Only for acute HPV TRPC6 is indispensable and cannot be compensated by up-regulation of other TRPC channels. Moreover, the lack of acute HPV in TRPC6−/− mice has profound physiological relevance, because partial occlusion of alveolar ventilation provoked severe hypoxemia in TRPC6−/− but not in WT mice (Weissmann et al. 2006).

We recently identified a novel mechanism of TRPC6 channel activation by redox signalling which is important for cellular toxicity by hypoxia in the lung (Weissmann et al. 2012). Lung ischemia-reperfusion-induced injury (LIRI) is a life-threatening condition that causes pulmonary edema in lungs intended for transplantation and can be induced by ischemia in isolated perfused mouse lungs from wild-type mice. Most interestingly, after 90 min of ischemia, edema was completely absent in isolated lungs from TRPC6-deficient mice. Edema did also occur in lungs deficient in NADPH oxidase 2 (NOX2), raising the possibility that the two proteins are members of the same signal transduction pathway. After identifying other proteins in pulmonary endothelial cells, we postulate a novel mechanistic model comprising Nox2-derived production of superoxide, activation of phospholipase C-γ, inhibition of DAG kinase, and DAG-mediated activation of TRPC6 which is essential in increasing endothelial permeability. In later stages fluid and immune cells can then enter the alveolar space inducing LIRI.

The NOX family consists of seven members, NOX1-5, and two dual oxidases (DUOX) DUOX1 and 2 (Altenhofer et al. 2012; Lassegue et al. 2012). After activation they produce superoxide anions which can form multiple forms of reactive oxygen species (ROS). NOX1 is most highly expressed in colon epithelium, while NOX2 is also known as gp91phox the enzymatic complex responsible for the phagocyte respiratory burst. NOX3 is not expressed in lung tissues but in the

inner ear and the cochlea of mice (Paffenholz et al. 2004). The NOX4 isoform is abundant in kidney cortex where it was originally discovered and NOX5 is the only isoform which can be activated by Ca^{2+} through its Ca^{2+}-binding EF hands at the amino terminus. Unfortunately NOX5 is not expressed in rodents and cannot be analyzed in mouse models. In the lung, NOX isoforms are expressed in invading inflammatory cells (NOX1, 2 and 4) epithelial cells (NOX1, 4 and DUOX1 and 2), endothelial cells (NOX1, 2, 4), smooth muscle cells (NOX2 and 4) and mesenchymal cells (NOX2 and 4) (Hecker et al. 2012). It is tempting to speculate that NADPH oxidases may also represent important cellular sensors for TRPC activation by inhaled toxins. Along these lines, recent evidence suggest that NOX isoforms are activated not only by inflammatory mediators, but also by toxic substances like cisplatin (Rybak et al. 2012), bleomycin (Carnesecchi et al. 2011) and paraquat (Cristovao et al. 2009). Cisplatin induced hearing loss is associated with higher levels of ROS and down-regulation of NOX3 prevents cisplatin ototoxicity (Rybak et al. 2012). NOX4-deficient mice are protected from bleomycin-induced lung fibrosis while inflammatory mediators are not changed in the mouse model (Hecker et al. 2012). The activation of NOX isoforms by toxins is best known for paraquat, a toxic herbicide in various tissues, including lung. Paraquat increased the expression of NOX1 in rat dopaminergic N27 cells (Bokemeyer et al. 1998). NOX1 isoforms transfer an electron to paraquat enabling the paraquat-cation to donate an electron to molecular oxygen, generating a superoxide anion (Bielefeld et al. 2005; Bus and Gibson 1984). Subsequently, the accumulation of ROS induces neurotoxicity in the substantia nigra which may result in Parkinson's disease (Cristovao et al. 2012).

Superoxide anions not only convert nitric oxide (NO) to $ONOO^-$ decreasing vasodilation in the vasculature, but also induce multiple signaling cascades in different cell types initiating transcription of inflammatory target proteins which can induce asthma, acute lung injury, lung fibrosis, COPD and lung cancer (Lee and Yang 2012). Moreover, superoxide anions produced by NOX isoforms are able to form hydrogen peroxide (H_2O_2) in a reaction with H_2O catalyzed by extracellular superoxide dismutase (SOD). Hydrogen peroxide is able to permeate the cell membrane to activate PLC isoforms which produce DAG from PIP_2. Furthermore, inhibition of DAG kinases which degrade DAG to phosphatidic acid (PA) by hydrogen peroxide will induce DAG accumulation stimulating TRPC3/6/7 channels whereby increasing intracellular Ca^{2+} concentrations. While moderate elevated Ca^{2+} levels increase cellular permeability by actin fiber formation resulting in cell shape changes, Ca^{2+} overload will induce cell death by necrosis or apoptosis (see Fig. 7).

Possible signal transduction pathways from NOX isoforms as toxicity sensor to TRPC3/6/7 activation and the resulting cellular effects are summarized in Fig. 7. In the future, it will be essential to dissect these signal transduction cascades in more detail to identify pharmacological targets to be exploited in acute and chronic toxicity.

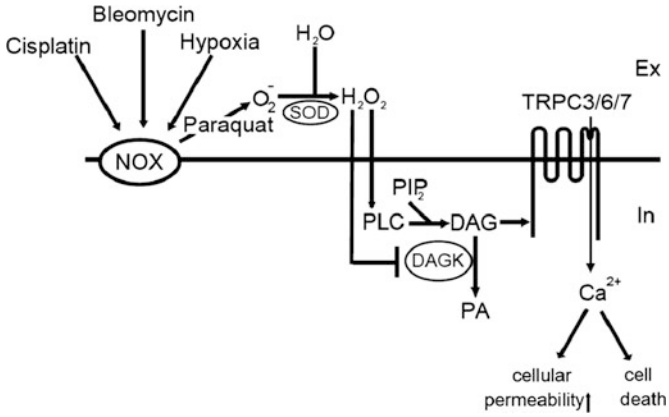

Fig. 7 Putative signalling mechanisms for toxin activation of TRPC channels. See text for details. *NOX* NADPH oxidase, *SOD* superoxide dismutase, H_2O_2 hydrogenperoxide, *PLC* phospholipase C, *DAG* diacyl glycerol, PIP_2 phosphatidylinositol 4,5-bisphosphate, *DAGK* diacyl glycerol kinase, *PA* phosphatidic acid, *Ex* extracellular, *In* intracellular (Modified from Weissmann et al. 2012)

11 Outlook: Respiratory Chemosensors and Targeted Therapy in Lung Toxicology

To summarize, the present synopsis describes the role of chemosensory TRP channels in the airways with regard to the detection of TIHs and the mediation of TIH-promoted adaptive or noxious effects. The fact that TRP channels represent specific targets of TIHs broadens our mechanistic understanding of toxic lung injury and has implications on novel therapeutic approaches in pulmonary toxicology.

TRP channels are expressed throughout the airways from nasal mucosa to the alveolo-capillary system. They have been found in neuronal cells, especially in nerve endings of C fibers, but also in non-neuronal cells, i.e. in the pulmonary epithelium, in smooth muscle cells of bronchi and vasculature as well as in pulmonary endothelial cells. Regarding the function of TRP channels (in particular TRPA1) in neuronal cells, a critical role as toxicant sensor has been established and activation of TRP channels in nerve endings of the airways leads to the stimulation of protective reflexes, but also to neurogenic inflammation. With respect to the role of pulmonary TRP channels in non-neuronal cells, TRPC channels expressed in vascular smooth muscle cells or pulmonary endothelium are the best characterized members. Pulmonary TRPC channels fulfill important functions in regulating pulmonary blood flow and are not directly activated by TIHs but subsequently to the activation of G protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs), in turn leading to the stimulation of phospholipase C. Furthermore, a role of TRPV channels (in particular TRPV4) in the respiratory epithelium has been elucidated. These channels are activated following stimulation of G_q coupled

receptors (e. g., protease activated receptors) and promote the release of inflammatory cytokines upon exposure towards TIHs thus contributing to perturbations of the epithelial barrier function.

Thus, according to a simplified model, pulmonary TRP channels can be subdivided into sensor TRP and effector TRP channels. The former (e.g. TRPA1) are mainly expressed in neuronal cells and are directly activated by TIHs. The latter (e.g. TRPV4) are mainly expressed in non-neuronal cells and represent a more distal effector of GPCR- or RTK-promoted signaling pathways. Of note, prototypical sensor TRPs, like TRPA1 have been recently also described in non-neuronal cells. However, their function in this cellular context is still less defined.

The point of view that TIHs act via defined signaling pathways originating from membrane receptors contrasts with previous models describing the action of TIHs merely as unspecific damages of critical biomolecules like membrane lipids or the DNA. According to the general opinion of unspecific effects promoted via TIHs the therapy of toxic lung injury relies on symptomatic efforts or the attempt to inactivate reactive inhalants by applying antioxidative substances or radical scavengers. Based on the view that specific molecules and signaling events are involved in toxic effects in the respiratory system, the potential of targeted therapeutic interventions arises. TRP channels are especially suited for targeted therapies since these molecules are exposed at the plasma membrane and thus, easily accessible for drugs. In fact, a number of modulators of TRP channels with relatively low systemic toxicity have been identified and are available for in vivo testing. Whereas the concept of targeted therapies in toxicology is relatively new, this approach is well established in cancer therapy. In analogy to the experience in oncology, specific drugs targeting TRP-promoted signaling events should be combined with symptomatic and unspecific approaches.

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The Cardiomyocyte Cell Cycle in Hypertrophy, Tissue Homeostasis, and Regeneration

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Abstract Mammalian cardiomyocytes withdraw from the cell cycle shortly after birth. Although the adult heart is unable to regenerate, numerous reports have shown that adult cardiomyocytes exhibit a dynamic range of cell cycle activity under various physiological and pathological conditions. Reason and consequence of cardiomyocyte cell cycle activity remain unclear and have led to a number of misconceptions. Understanding the scenarios in which cycling happens may promote new perspectives on the differentiated state of cardiomyocytes, treatments for hypertrophy, heart regeneration and cancer therapy. In this review we discuss the result of cardiomyocyte cell cycle activity in aging and disease and studies manipulating cardiac cell cycle activity to promote cardiac regeneration. In addition, we focus on cardiomyocyte differentiation, cell cycle exit, and the relationship between ploidy and regenerative potential. Finally, we provide observations that may further advance the goal of inducing adult mammalian heart regeneration through cardiomyocyte proliferation.

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Contents

1 Cardiomyocyte Proliferation vs. Cardiac Stem Cells

Many decades ago Rumyantsev discussed the "interrelations of the proliferation and differentiation processes during cardiac myogenesis and regeneration" (Rumyantsev 1977). At this time he argued against a cardiac stem cell population and suggested based on the observation of cardiac cell cycle activity during disease that cardiac regeneration might be possible by inducing cardiomyocyte proliferation. Great effort has been invested to understand cardiomyocyte cell cycle control during development and disease, which has been reviewed in detail in 2000 (MacLellan and Schneider 2000). Since then the field has shifted due to the "identification of cardiac progenitor cells capable of giving rise to cardiac myocyte-like cells" (Ahuja et al. 2007). However, the potential of cardiac and non-cardiac stem/progenitor cells remains controversial (Neri et al. 2010; Reinecke et al. 2008). In recent years, a number of observations have promoted resurgence in the idea of "heart regeneration through cardiomyocyte proliferation" (Mercola 2012; Kajstura et al. 2012). Therefore, this review will focus on the current knowledge of cell cycle control of cardiomyocytes in hypertrophy, tissue homeostasis, and regeneration.

2 Cardiomyocyte Cell Cycle in the Developing Heart

2.1 Cell Division vs. Binucleation

The heart is formed from rapidly proliferating precursor cells whose proliferation rate drops upon differentiation into cardiomyocytes. The cardiomyocytes form the linear heart tube, a slowly proliferating structure. Subsequently, the heart loops and the regional activation of an increase in cellular size, followed by an increase in the rate of proliferation, initiates chamber formation (Soufan et al. 2006; de Boer et al. 2012; Martin-Puig et al. 2008). Subsequent fetal mammalian heart growth occurs by continuous cardiomyocyte proliferation. In contrast, postnatal heart growth occurs primarily by cardiomyocyte enlargement; often called physiological hypertrophy (for discussion see [Dorn et al. 2003]).

Mammalian cardiomyocytes exhibit two temporally distinct cycling phases (Li et al. 1996; Soonpaa et al. 1996; Jonker et al. 2007a). The first cycling phase occurs in the fetal heart and results in cell division. The rate of proliferation declines as gestation progresses, with the vast majority of cardiomyocytes entering a quiescent state at birth. The second cycling phase occurs in the neonatal heart and results in cellular binucleation through cytokinesis failure (Li et al. 1997). Although cardiomyocytes withdraw from the cell cycle in conjunction with binucleation, they maintain the ability to re-enter the cell cycle under a variety of homeostatic and pathological conditions. The underlying mechanism of this switch from hyperplastic to hypertrophic growth is still elusive but it appears to be highly associated with the onset of binucleation. Binucleation is seen in cardiomyocytes of all mammalian species, however, its timing differs slightly between species; beginning in the last third gestation in sheep (Jonker et al. 2007b) and human (Kim et al. 1992), and shortly after birth in mouse (Li et al. 1996) and rat (Soonpaa et al. 1996).

2.2 Cell Cycle Exit: CIP/KIP vs. INK4

Cell cycle exit in most cell-types is primarily mediated by the CIP/KIP (i.e. $p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$) and INK4 (i.e. p16INK4a, p15INK4b, p18INK4c and p19INK4d) family of cyclin-dependent kinase inhibitor (CDKI) proteins (Vidal and Koff 2000). CDKIs regulate the cell cycle by inhibiting CDK activation either by binding to monomeric CDKs or disrupting Cyclin-CDK complexes. Whereas INK4 family members specifically regulate CDK4/6 activity, CIP/KIP family members regulate a broader range of CDKs (Sherr and Roberts 1999).

Mammalian cardiomyocyte cell cycle exit during fetal and neonatal phases correlates with the up-regulation of both $p21^{CIP1}$ and $p27^{KIP1}$ (Horky et al. 1997; Poolman et al. 1998; Burton et al. 1999b). Furthermore, the expression of both $p21^{CIP1}$ and $p27^{KIP1}$ persists in the adult heart (Koh et al. 1998). Observations whereby (i) deletion of $p27^{KIP1}$ abrogates cell cycle withdrawal postnatally and (ii) down-regulation of $p27^{KIP1}$ and

$p21^{CIP1}$ in varied pathological states (reviewed in Sect. 3.1) indicate that these CDKIs contribute to cardiomyocyte cell cycle withdrawal.

In contrast to $p21^{CIP1}$ and $p27^{KIP1}$, $p57^{KIP2}$ is expressed exclusively in the fetal mammalian heart (Nagahama et al. 2001). Relative to $p21^{CIP1}$ and $p27^{KIP1}$, the function of $p57^{KIP2}$ is less well known. However, unlike $p21^{CIP1}$ and $p27^{KIP1}$, which are well known to regulate cell cycle exit, $p57^{KIP2}$ activity appears to be more synonymous with processes related to cell differentiation (Pateras et al. 2009). During mid-gestational mammalian heart development, $p57^{KIP2}$ expression is restricted to cardiomyocytes of the inner trabecular layer (Kochilas et al. 1999). This spatial restriction of $p57^{KIP2}$ is consistent with its general role in cell differentiation as the trabecular layer is ultrastructurally more differentiated and cardiomyocytes from this layer are less proliferative than other layers (i.e. the outer compact layer) of the heart (von Gise et al. 2012; Pasumarthi and Field 2002). However, there is also evidence that $p57^{KIP2}$ contributes to cardiomyocyte cell cycle exit. Splotch mice (which contain a mutation in Pax3) exhibit a thinned myocardium (Conway et al. 1997) and high expression of $p57^{KIP2}$ in both trabecular and compact layers (Kochilas et al. 1999). Furthermore, Cardiomyocyte-specific transgenic expression of FOXO1 during heart development causes embryonic lethality at embryonic day E10.5 due to severe myocardial defects that coincide with premature activation of $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ and decreased cardiomyocyte proliferation (Evans-Anderson et al. 2008). Collectively, these observations correlate up-regulation of $p57^{KIP2}$ with loss of cardiomyocyte proliferative-competence. Whether loss of proliferative-competence is due to precocious differentiation, cell cycle exit, or both is unclear.

In contrast to the CIP/KIP family, INK4 family members do not appear to be significantly expressed in fetal or neonatal mammalian hearts and thus are unlikely to regulate cardiomyocyte cell cycle exit during development (Kajstura et al. 2000; Koh et al. 1998; Zindy et al. 1997). The role of INK4 family members in maintenance of cardiomyocyte cell cycle exit in the adult heart, however, is less clear. Whole heart tissue Western analysis failed to detect any significant protein levels of the INK4 family members in 4-week or 15-month old mouse hearts (Zindy et al. 1997). In contrast, Immunofluorescence-based analysis indicated that 9 %, 11 %, 25 %, and 82 % of cardiomyocytes isolated from 1-day, 4-, 12-, and 27-month old rat hearts, respectively, are positive for p16INK4a (Kajstura et al. 2000). Despite the lack of a definite consensus with regards to INK4 expression in the aging heart, the relative absence of p16INK4a in the young heart, when cardiomyocytes undergone developmentally regulated cell cycle withdrawal, questions the function of p16INK4a expression in adult cardiomyocytes. While, speculative, the onset of p16INK4a expression in cardiomyocytes may be related to (i) the absence of adult cardiomyocyte-born tumors (reviewed in Sect. 5.2) and (ii) the reduced ability of the aging heart to undergo compensatory, cell-cycle-mediated, hypertrophy in response to pathological stimuli (reviewed in Sect. 3.2).

2.3 Cytokinesis Failure Causes Binucleation

The molecular mechanisms that result in cardiomyocyte binucleation are unknown. Immunofluorescence analysis indicated that binucleating cardiomyocytes progress properly through all stages of mitosis (i.e. prophase, metaphase, anaphase, and telophase). However, while cardiomyocytes appear to form a contractile acto-myosin ring during anaphase and undergo cleavage furrow ingression during telophase, the ingressed furrow fails to promote abscission (Li et al. 1997; Engel et al. 2006b).

Furrow ingression requires the localization and interaction of numerous factors to the division plane where the acto-myosin ring forms (D'Avino et al. 2005). Amongst these factors is Anillin, which functions as a scaffold to stabilize proteins such as RhoA and CD2AP required for furrow ingression (D'Avino 2009; Piekny and Maddox 2010). In binucleating cardiomyocytes, Anillin exhibits an atypical diffused localization at the division plane (Engel et al. 2006b). What causes this atypical Anillin localization is unknown. Another phenotype of binucleating cardiomyocytes is asymmetric furrow ingression, whereby the furrow ingresses only from one side of the cell (Engel et al. 2006b). The relationship between Anillin mislocalization and asymmetric furrow ingression is unclear. Furthermore, why asymmetric furrow ingression is unable to promote abscission is also unknown.

While cytokinesis failure is highly associated with the loss of proliferative-competence in cardiomyocytes, not all cell-types follow this paradigm. Liver hepatocytes also binucleate during postnatal development by cytokinesis failure. However, unlike cardiomyocytes, hepatocytes appear to maintain some level of proliferative- and regenerative-competence (Gentric et al. 2012). This has led many to speculate that the key to adult cardiomyocyte proliferative-competence lies within hepatocyte biology. While possible, it should be noted that unlike cardiomyocytes, hepatocytes do not appear to undergo furrow ingression during binucleation (Guidotti et al. 2003; Margall-Ducos et al. 2007). Thus, the underlying mechanism of binucleation and subsequent cell cycle exit may be fundamentally different between cardiomyocytes and hepatocytes.

2.4 Physiological Relevance of Binucleation

While the timing of binucleation in mammals correlates highly with the onset of cardiomyocyte cell cycle withdrawal, the percentage of binucleation varies amongst mammalian species: ~90 % in rodents (Li et al. 1996; Soonpaa et al. 1996), ~70 % in sheep (Burrell et al. 2003; Thornburg et al. 2011), and ~23–57 % in humans (Olivetti et al. 1996; Schmid and Pfitzer 1985; Kajstura et al. 2012). What accounts for interspecies differences in the percentage of binucleated cardiomyocytes is unknown. The observation of precocious cardiomyocyte binucleation in fetal hearts under increased hemodynamic stress suggests that the percentage of binucleated cardiomyocytes may be a function of species-specific hemodynamic demand (Jonker et al. 2007b, 2010). While little is known with regards to the functional

relationship between percentage of binucleated cardiomyocytes and hemodynamic demand it is known that (i) hypertrophic growth is a function of hemodynamic demand and (ii) the extent of hypertrophic growth in cardiomyocytes and vascular smooth muscle cells appears to be positively correlated with increased ploidy (Owens and Schwartz 1983; Brodsky and Uryvaeva 1977). Thus, variations in the percentage of binucleated cardiomyocytes between mammalian species may reflect species-specific requisites in hypertrophic potential to accommodate fluctuations in hemodynamic demand.

In mammalian cardiomyocytes, expression profiles of the major cyclins and their respective CDKs correlate with both fetal and neonatal cell cycling (MacLellan and Schneider 2000). To date, the only exception with regards to differential expression of cyclins during development is Cyclin G1, which is up-regulated during the second, neonatal, cell cycle phase (Liu et al. 2010b). In non-myocytes, Cyclin G1 is up-regulated after Spindle Assembly Checkpoint-induced mitotic arrest (Russell et al. 2012). The up-regulation of Cyclin G1 in this scenario appears to be a pro-survival response to post-mitotic arrest. Cyclin G1 knockout and over-expression studies in the heart have shown that Cyclin G1 promotes cardiomyocyte polyploidization (Liu et al. 2010b). Thus, rather than promoting binucleation through cytokinesis failure, Cyclin G1 may promote viability of cardiomyocytes after cytokinesis failure.

2.5 Relationship Between Cell Cycle Exit and Differentiation

Cellular differentiation describes the transition to a more highly specialized state. Exit from the cell cycle is often essential for cell differentiation (Budirahardja and Gonczy 2009). How cell cycle exit and differentiation are linked is unclear and appears to vary among cell-types. For example, in skeletal muscle, proliferation of myoblasts and their differentiation into contractile myotubes is mutually exclusive (Andres and Walsh 1996). In contrast, fetal cardiomyocytes proliferate during development even though they already contain a contractile apparatus and actively contract (van Amerongen and Engel 2008). Thus, cardiomyocyte function (i.e. cell contraction) does not require cell cycle exit. This makes the definition of "cardiomyocyte differentiation" difficult as differentiation appears to be a continuous process that is associated with a continued decrease in proliferative-competence. Therefore, one finds in the literature often the term "terminal differentiation" to define the state when cardiomyocytes are fully matured (i.e. full sarcomere differentiation enabling maximal contraction force) and permanently withdrawn from a proliferative state. As discussed in the following sections the term "terminal" is confusing as adult cardiomyocytes can re-express fetal genes and re-enter the cell cycle, albeit not divide.

Binucleation, hypertrophic growth potential, increased organization of myofibrillar ultrastructure, and cell cycle withdrawal, are all criteria used to describe the differentiated state of an adult cardiomyocyte. Additionally, acquisition of an adult-like differentiated state is associated with changes in expression of a panel of genes, collectively called 'the fetal gene program' which include: (i) isoform switching of genes required for metabolism (e.g. GLUT1 to GLUT4) (Wang and Hu 1991) and

sarcomere components (e.g. slow skeletal Troponin I to cardiac Troponin I) (Siedner et al. 2003) and (ii) decreased expression of atrial natriuretic factor (ANF) (Claycomb 1988).

Cardiomyocytes are often considered fully or terminally differentiated after the second, neonatal, cycling phase, which coincides with the onset of adult-like differentiation markers stated above. However, whether cell cycle exit during the first, fetal, cycling phase is required for a transition from a fetal to an adult gene program remains unclear. Hearts of $p27^{KIP1}$ knockout mice exhibit postnatal cardiomyocyte proliferation (Poolman et al. 1999). However, although adult $p27^{KIP1}$ knockout cardiomyocytes eventually exit the cell cycle they continue to express markers of the fetal gene program (e.g. α-skeletal actin and ANF) (Poolman et al. 1999). Similarly, postnatal hearts of transgenic mice with cardiomyocyte-specific overexpression of CDK2 exhibit a decreased percentage in binucleated cardiomyocytes, indicating incomplete progression to the second cell cycling phase, and continue to express markers of the fetal gene program (e.g. β-MHC and ANF) (Liao et al. 2001). Collectively, these observations suggest that proper exit from the first, fetal, cell cycle phase may be required for the fetal- to adult-gene program switch.

3 Cardiomyocyte Cell Cycle in the Adult Heart

3.1 Hypertrophy: Are Cardiomyocytes Truly Withdrawn from the Cell Cycle?

Cardiac hypertrophy can be subdivided into physiologic and pathologic forms (Abel and Doenst 2011; Bernardo et al. 2010). Physiologic hypertrophy occurs in response to exercise and pregnancy. In contrast, pathologic hypertrophy occurs in response to pressure overload (e.g. aortic stenosis), volume overload (e.g. mitral regurgitation), certain growth factors, cytokines, hormones, and changes in the extracellular matrix (Kehat and Molkentin 2010; Frey and Olson 2003). In contrast to physiologic hypertrophy, pathologic hypertrophy results in fetal gene re-expression, cardiomyocyte apoptosis and fibrosis and, after a period of compensatory function, progressive decline in left ventricular performance and, ultimately, heart failure (Bernardo et al. 2010).

In the kidney, tubular cell hypertrophy can be either cell cycle-independent (which occurs by inhibition of protein degradation by lysosomal enzymes) or cell cycle-dependent (which occurs by G1/S cell-cycle arrest) (Liu and Preisig 2002). With regards to cell cycle-dependent growth, it is well known that cells grow in size during the cell cycle, with the greatest degree of growth occurring during G1 phase. Evidence that growth can occur in cell cycle-arrested cells is observed in budding yeast mutants, whereby inhibition of S phase transition by mutation of cdc28/CDK2 results in an increase of cell size ~7-fold (Goranov et al. 2009).

Mammalian cardiomyocyte growth during postnatal development (physiological hypertrophy) appears to be a function of cell cycle-independent growth. This is supported by the absence of cell cycle markers during postnatal development after

binucleation (Soonpaa and Field 1998; Ikenishi et al. 2012; Walsh et al. 2010) despite a progressive increase in cardiomyocyte size and mass (van den Hoff et al. 1997; Bishop and Hine 1975; Fraticelli et al. 1989). However, in response to pathologic cardiac stress, cardiomyocytes appear to undergo a transition from cell cycle-independent to cell cycle-dependent hypertrophic growth. This is evidenced by the increased expression of G1 phase pro-cycling factors under pathological conditions (Brooks et al. 1997; Li et al. 1998; Busk and Hinrichsen 2003). For example, cardiac pressure overload-induced hypertrophy in rodents induces the up-regulation of early response genes, such as c-Myc and c-Fos, and delayed-response genes, such as Cyclin D (Izumo et al. 1988). Consistent with G1 cell cycle activity, pressure overload-mediated hypertrophy results in up-regulation of CDK2 (Zhong et al. 2006) and phosphorylation of Rb (Angelis et al. 2008). Reciprocally, and consistent with cell cycle entry, pressure overload-mediated hypertrophy decreases the expression of $p21^{CIP1}$ and $p27^{KIP1}$ (Li and Brooks 1997). These changes in the expression of cell cycle regulators are recapitulated in clinical forms of human heart failure (Burton et al. 1999b). Furthermore, studies utilizing knockout mouse strains and pharmacological inhibitors have shown that the activity of many of these cell cycle regulators is required for pathologic hypertrophic growth. For example, c-Myc is required for transaortic constriction-induced hypertrophic growth (Zhong et al. 2006), delivery of $p27^{KIP1}$ fusion protein suppresses myocardial infarction (MI)-induced cardiac remodeling (Konecny et al. 2012), and overexpression of $p21^{CIP1}$ prevents serum-induced protein synthesis and fetal gene expression (i.e. skeletal α-Actinin and ANF) in cultured neonatal cardiomyocytes (Tamamori et al. 1998). In contrast to G1 phase activity, S phase activity appears not to be as prevalent as only a minor fraction of cardiomyocytes exhibit DNA-synthetic activity in response to various pathologic insults (Soonpaa and Field 1998). Collectively, these observations indicate that cardiomyocytes undergo cell cycle-dependent hypertrophic growth under pathological stress.

Previously, pathologic hypertrophy has been described as a failed attempt by cardiomyocytes to regenerate the heart after injury. Indeed, hypertrophic cardiomyocytes under pathological stress phenotypically resemble and exhibit cycling characteristics of cardiomyocytes initiating proliferation; as they (i) enter the cell cycle (see previous paragraph), (ii) re-express the fetal gene program (Rajabi et al. 2007), and (iii) exhibit myofibrillar disorganization (reviewed in Sect. 3.5)(Machackova et al. 2006). These data suggest that limited compensatory function of physiological hypertrophy promotes cardiomyocytes to switch to cell cycle-dependent growth, which may also represent a de facto regenerative response, albeit an unsuccessful one. How these events relate to the transition of maladaptive heart failure is unknown.

3.2 Compensatory Hypertrophy: A Hyperplastic S/G2 Block?

Adult mammalian cardiomyocyte cell cycle entry during compensatory hypertrophy is associated with reduced expression of $p21^{CIP1}$ and $p27^{KIP1}$ (Li and Brooks 1997) and, interestingly, a reciprocal increase in the expression of $p57^{KIP2}$

(Burton et al. 1999b). The relationship between $p57^{KIP2}$ and cell cycle-mediated hypertrophy is unclear. However, there is evidence to suggest it is cell cycle-based and may limit progression though the G2/M checkpoint into mitosis: Trophoblast giant cells do not progress through mitosis but are capable of multiple rounds of endoreduplication (i.e. multiple S phases without an intervening mitosis). $p57^{KIP2}$ punctuates the completion of DNA replication in trophoblast giant cells (Hattori et al. 2000). These observations suggest that perhaps one function of $p57^{KIP2}$ in adult mammalian cardiomyocytes undergoing pathological hypertrophy is to inhibit entry into mitosis.

3.3 Do CDKIs Differentially Control Hypertrophic Potential?

The preferential utilization of CIP/KIP family CDKIs to regulate cell cycle exit is not unique to cardiomyocytes, as regulation of cell cycle exit by INK4 family members in podocytes, vascular smooth muscle cells, and adipocytes is conspicuously absent in the literature. Interestingly, like cardiomyocytes, these cell-types are also capable of undergoing hypertrophic growth (Ruster et al. 2008; Braun-Dullaeus et al. 1999; Cooke and Naaz 2004; Fine and Norman 1992). Furthermore, to our knowledge, there are no cell-types described in the literature that undergo endogenous cell cycle-mediated hypertrophy by way of utilizing INK4 family members to regulate cell cycle exit.

Why cell cycle regulation through CIP/KIP family members correlates with hypertrophic growth potential and whether INK4 family members act differently is unclear. However, unlike CIP/KIP family members, up-regulation of p16/INK4a is a key event in the induction of replicative senescence (Alcorta et al. 1996). Interestingly, a progressive increase in the frequency of p16INK4a-positive rat cardiomyocytes has been observed in the aging rat (i.e. 11 % at 4 months, 82 % at 27 months of age) (Kajstura et al. 2000). This increase in cardiomyocyte INK4a expression inversely correlates with the ability of the adult rat heart to sustain compensatory hypertrophy with age (Isoyama et al. 1987, 1988). Thus it would be interesting to determine whether p16INK4a limits the ability of a cardiomyocyte to undergo cell cycle-dependent hypertrophy. In cultured neonatal rat cardiomyocytes, ectopic expression of p16INK4a inhibits induction of the fetal gene program (Tamamori et al. 1998), a marker of compensatory hypertrophy, offering some credibility that p16INK4a expression may abrogate compensatory, cell cycle-mediated, hypertrophic potential.

3.4 Cardiac Homeostasis: Is the Heart Truly a Post-Mitotic Organ?

Many tissues undergo cell turnover under physiological conditions (Pellettieri and Sanchez Alvarado 2007). Although historically considered to be a post-mitotic organ, over the past decades, low levels of DNA synthesis activity have been observed in

cardiomyocytes in the postnatal rat and mouse heart under normal physiological growth, suggesting that the adult heart may undergo cell turnover (Soonpaa and Field 1998; Soonpaa et al. 2012). Recently, based on carbon-14 incorporation into DNA, two independent labs have found evidence for cardiomyocyte renewal in the human heart (Kajstura et al. 2012; Bergmann et al. 2009). However, the rate and kinetics of renewal reported by the two labs varied significantly. Bergmann et al. show a decrease in cardiomyocyte renewal over time with 1 % new cardiomyocytes/year at the age of 25 % and 0.45 % new cardiomyocytes/year at the age of 50. In contrast, Kajstura et al. show an increase in cardiomyocyte renewal over time with 7 % new cardiomyocytes/year from ages 20 to 40 and 8 %, 10 %, 14 %, and 19 % new cardiomyocytes at ages 50, 60, 70, and 80, respectively. These discrepancies are potentially due to different methods of dating (i.e. forward vs. retrospective carbon-14 dating), low sample numbers, assumptions in different mathematical calculations, differences in cardiac nuclei sources (frozen tissue vs. fresh tissue harvested within 24 h of organism death), and high variability amongst samples. For example, in the study by Kajstura et al., cardiomyocyte nuclei of normal hearts from two 68- and one 69-year old individual differ significantly in their ploidy according to the provided FACS plots in the supplemental data. A general issue that pertains to both studies is the rate of cardiomyocyte binucleation over time. The average frequency of cardiomyocyte binucleation in post-mortem human hearts of different ages remains relatively constant over time (i.e. ~25 %, with notable large degree of error) (Olivetti et al. 1996; Kajstura et al. 2012). However, it is currently not possible to discount that binucleation can increase over the lifespan of a particular individual. Thus, based on the method of analysis (i.e. carbon-14 levels in isolated cardiomyocyte nuclei) binucleation would be misinterpreted as cardiomyocyte renewal. To date, these studies are highly controversial and have not been reproduced independently. Thus, even though these data suggest the exciting possibility that enhancement of endogenous homeostatic growth may promote cardiac regeneration we urge a critical analysis of data regarding this hot topic to avoid misinterpretations and hasty conclusions.

While these studies indicate that new cardiomyocytes are generated during adulthood, the source of these new cardiomyocytes is unclear. Are they generated from pre-existing cardiomyocytes, progenitor cells, or stem cells? Kajstura et al. speculate based on histological analyses of cell cycle markers (Ki67, phosphorylated Histone H3 (S10), and Aurora B, which indicate cell cycle progression, entry into mitosis, and progression through cytokinesis, respectively) that a significant degree of new cardiomyocytes may be generated by proliferation of cardiomyocytes. However, it should be noted that while the cycling events associated with these markers are necessary for cell division they are not sufficient. This is a very important point as cell cycle activity in adult cardiomyocytes is associated not only with proliferation but also polyploidization and binucleation (Bersell et al. 2009). Thus, we would like to clarify here what these markers signify utilizing the recent paper by Kajstura et al. as an example. It is often noted that Ki67 is not a marker of ploidy formation. However, cycling giant trophoblasts (Zybina et al. 2004), as well as adult mammalian cardiomyocytes (Hesse et al. 2012) undergoing endoreduplication, and not cell division, are Ki67-positive. Thus, Ki67 signal can be

present in both proliferative and non-proliferative cell cycles, and thus should not be used as a marker to measure proliferation. In addition, Aurora B localized at the midbody – which forms as a result of cleavage furrow ingression – has been introduced to the cardiac field as unequivocal evidence of cardiomyocyte cytokinesis. However, while Aurora B midbody-localization is a common marker of furrow ingression and late-stage cytokinesis, it is not proof of abscission (i.e. cell division). In other cell-types it has been shown that numerous defects can result in abscission failure and furrow regression even though an Aurora B-positive midbody has formed (Steigemann et al. 2009; Carlton et al. 2012). Furthermore, binucleating mammalian cardiomyocytes have an Aurora B-positive midbody (Engel et al. 2006b) and central spindle remnants persist temporarily in binucleated vascular smooth muscle cells (Nagata et al. 2005). Additionally, one finds in the literature often some peculiar Aurora B stainings for cardiomyocytes such as homogenously labeled condensing anaphase chromosomes (Kajstura et al. 2012). Aurora B, as other chromosomal passenger complex proteins such as Survivin, regulates kinetochore-microtubule attachment and central spindle midzone formation at anaphase (Hu et al. 2012; van der Waal et al. 2012). Thus, Aurora B association with the kinetochore during pro-metaphase and metaphase appears as discrete foci (reflecting the number of kinetochores in the nucleus) and association with the central spindle at anaphase appears as thread-like structure perpendicular to the cleavage plane (Keen and Taylor 2004). Finally, we would like to note that the phosphorylation at serine 10 of histone H3 is lost from chromosomes at mid-anaphase, not telophase (Hirota et al. 2005). In conclusion, given the apparent lack of knowledge with regards to the biology of cell cycle markers used, the accuracy of published immunohistological-based quantitation for various mitotic events in the field of cardiac regeneration and the conclusions drawn from these data is of some concern.

The hypothesis that cardiomyocyte renewal occurs in the aging heart is further substantiated by a recent report investigating cardiac homeostasis in mice (Senyo et al. 2012). The rate of renewal was found to be consistent with that found in humans in the Bergmann et al. study (i.e. 0.75 % new cardiomyocytes/year). Although these data collectively support the notion that the mammalian heart is capable of tissue homeostasis and that adult pre-existing cardiomyocytes likely contribute to this event, conclusive evidence that the newly formed cardiomyocytes arise from pre-existing cardiomyocytes remains to be shown. Additionally, it is worth noting, even if cardiomyocyte renewal occurs, the rate at which it occurs is ultimately not high enough to overcome age-associated atrophy or cardiomyocyte loss due to injury. In conclusion, it is important to conclusively demonstrate that cardiomyocyte renewal indeed occurs and to identify the cardiomyocyte phenotype that permits homeostasis. This knowledge may help to harness the endogenous proliferative potential of cardiomyocytes to develop strategies to cure heart disease by reversing cardiac injury after pathological insult.

3.5 The Concept of Dedifferentiation

Assuming that pre-existing cardiomyocytes can give rise to new cardiomyocytes during tissue homeostasis in the adult heart, it remains unclear if all adult cardiomyocytes are equally capable to contribute to homeostasis or whether there exists a subset of proliferation-competent cells. A reasonable assumption would be that diploid mononucleated cardiomyocytes have a higher proliferation capacity than binucleated cardiomyocytes as onset of binucleation is associated with loss of proliferative-competence (reviewed in Sect. 3.1). To date, there is no evidence or marker available to determine the proliferation potential of any given cardiomyocyte. Furthermore, it is also unclear by which mechanism pre-existing adult cardiomyocytes re-enter a proliferative/homeostatic state; do they undergo some form of dedifferentiation to a more fetal-like or even stem cell state, or do they already exist in a fetal-like state?

Proliferation is not generally associated with highly-specialized cell-types. Therefore, many differentiated cell-types are generally believed to undergo some extent of dedifferentiation to a less-specialized, or precursor, cell state to acquire proliferative competence (Poling et al. 2012; Heinen et al. 2012). Given this, it has long been suggested that cardiomyocytes require dedifferentiation to proliferate (Rumyantsev 1977). However, to date, there is no consensus as to what defines a dedifferentiated cardiomyocyte.

Previously, the re-expression of the fetal gene program has been considered as dedifferentiation and was seen in pathological hypertrophy as evidence for an attempt of the diseased heart to regenerate. Detailed expression analyses have however demonstrated that only a subset of fetal genes is re-expressed that play roles in metabolic and contractile functions and their respective transcription factors (Barry et al. 2008; Depre et al. 1998; Taegtmeyer et al. 2010). Thus, the re-expression of the fetal gene program is considered as an adaptive process to hemodynamic and metabolic stress.

Oncostatin M has recently been shown to induce the fetal gene program in postnatal cardiomyocytes and to promote growth factor-induced DNA synthesis (Kubin et al. 2011). These data suggest that induction of dedifferentiation, as determined by re-expression of the fetal gene program, increases the proliferation potential of cardiomyocytes. This is in accordance with the hypothesis derived from developmental studies that proliferation and differentiation potential are inversely correlated. Interestingly, in addition to the classical "fetal genes" as ANP and α-smooth muscle actin they identified several new markers of cardiomyocytes dedifferentiation such as α-smooth muscle actinin, destrin, Runx1, and c-kit. However, these data allow no conclusion regarding whether dedifferentiation in terms of fetal gene re-expression is a prerequisite of cell cycle re-entry or proliferation. Yet, there is evidence to suggest that the fetal gene program is a function of cell cycle re-entry. In neonatal rat cardiomyocytes, overexpression of $p21^{CIP1}$ inhibits serum-induced hypertrophy and the expression of skeletal α-Actinin and ANF (Tamamori et al. 1998). In addition, the intravenous delivery of a $p27^{KIP1}$ fusion protein inhibits MI-induced cardiac

hypertrophy as well as the expression of ANP in vivo (Konecny et al. 2012). Collectively, these observations suggest that dedifferentiation (e.g. re-expression of fetal gene program) is not a prerequisite, but rather a consequence of cardiomyocytes entering the cell cycle.

Besides induction of the fetal gene program, sarcomere disassembly has often been cited as indication that cardiomyocytes are proliferating, have undergone dedifferentiation, and are capable of cell division (Jopling et al. 2011). However, the sarcomere is fully compatible with cardiomyocyte proliferation. This is evidenced by the fact that fetal (Ahuja et al. 2004), neonatal (Engel et al. 2005, 2006b), and adult cardiomyocytes (Engel et al. 2005) exhibiting a functional contractile sarcomeric apparatus can undergo cell division. Given that the cytoskeleton undergoes extensive reorganization upon entry into mitosis (Heng and Koh 2010), the observed disassembly of the sarcomeres in cardiomyocytes during mitosis is likely an inevitable event (i.e. sarcomere disassembly is a natural result of entry into mitosis). This is supported by the fact that treatment of cardiomyocytes with tubulin and actin destabilizing compounds permits reversible disassembly of the sarcomere (Rothen-Rutishauser et al. 1998). Thus, sarcomere stability appears to be highly dependent upon the underlying cytoskeletal organization. It has often been hypothesized that cardiomyocyte binucleation results from failure of the sarcomere to fully disassemble during mitosis thereby presenting a mechanical barrier to furrow ingression and abscission (Li et al. 1997). To date, however, there is no evidence to support this hypothesis (Engel et al. 2006b). Collectively, the notion that sarcomere disassembly is a sign of cardiomyocyte dedifferentiation and a prerequisite for mitosis is premature.

4 Heart Regeneration

4.1 Fetal Heart Regeneration or Compensatory Growth?

In contrast to the adult mammalian heart, genetic and mechanical injury models indicate that the fetal heart exhibits significant regenerative capacity. Cardiomyocyte-specific inducible deletion of a Chromosome X-linked gene encoding holocytochrome c synthase, which is required for cell viability, ablates 50 % of fetal cardiomyocytes in male E13 mouse embryos (Drenckhahn et al. 2008). By late-gestation the injured hearts exhibited almost complete recovery, both in morphology and size. A relative increase in DNA synthesis (BrdU incorporation) and mitosis (phosphorylated histone H3(S10)) in cardiomyocytes of injured compared to non-injured hearts suggested that proliferation of pre-existing cardiomyocytes contributed to regeneration. Although contribution of stem cells and/or cardiac progenitor cells could not be ruled out in the regenerative response, fetal heart growth does not appear to be stem cell-based (Meilhac et al. 2003). Support for the significant regenerative capacity of the fetal heart is also observed with a MI injury model in early gestational sheep. Four weeks after MI, regeneration was completed and heart function recovered. Heart regeneration was accompanied by increase in DNA synthesis in

cardiomyocytes indicating extensive cardiomyocyte proliferation (Herdrich et al. 2010). Collectively, these observations indicate that the fetal heart has significant regenerative capacity and that cardiomyocyte proliferation contributes to this process. Given that polyploidization and binucleation are not features associated with fetal cardiomyocyte cycling, in contrast to postnatal cardiomyocyte cycling, DNA-synthetic and mitotic indices seem to be adequate criteria to determine proliferation in fetal cardiomyocytes.

While it is clear that the fetal heart can regenerate, both studies were performed at a stage in fetal development when endogenous proliferation indices are relatively high. To date no in vivo reports exist to measure the regenerative capacity of fetal hearts in later stages of gestation, when cardiomyocyte mitotic indices are relatively low (Soonpaa et al. 1996). However, this issue has been addressed in an in situ injury model (Blewett et al. 1997). Injury was induced by incision of the right ventricle of isolated mouse hearts from mid- and late-stages of gestation and wound healing was monitored at varying time points. Injured E14 hearts were capable of fully healing the incision with no scar formation, consistent with in vivo studies whereby mid-gestation injury was accompanied by extensive regeneration. However, the wound healing capacity was found to decrease when injury was induced later in gestation; E18 hearts showed partial healing (defined as incomplete bridging of the wound site by cardiomyocytes) with no scar formation and late gestation E22/birth hearts were repaired by scarring and collagen deposition. It is possible that the absence of systemic regenerative factors negatively affected hearts from later gestational time points to undergo scarless wound healing. However, organ growth appears to be generally mediated by local rather than paracrine factors (Lui and Baron 2011). This suggests that the absence of incision-induced wound healing may be due to an intrinsic loss of regenerative capacity of the heart. Furthermore, the incision wound healing study proposes the idea that regeneration in the heart may simply be a function of cardiomyocyte proliferative capacity at the developmental stage when injury occurs. Indeed, the proliferative capacity of isolated fetal rodent cardiomyocytes in culture decreases with age of isolation (Burton et al. 1999a). Thus, fetal heart regeneration appears to be rather a form of compensatory proliferation.

4.2 Heart Regeneration Based on Proliferation-Competent Cardiomyocytes

Although the second, neonatal, cell cycle phase in vivo is generally associated with withdrawal from the cell cycle, there is evidence that cardiomyocytes of postnatal day 1 (P1) mouse hearts in vivo undergo some proliferation prior to their terminal cell cycle and subsequent binucleation (Soonpaa et al. 1996). Similarly, in culture conditions, cardiomyocytes isolated from P1 neonatal rat hearts appear to maintain some proliferative-competence (Kajstura et al. 1994). These observations raised the question whether mammalian cardiomyocytes of the early postnatal heart are

capable of cardiac regeneration. In P1 rat hearts, both apex resection and MI injury models result in scarless regeneration due to cardiomyocyte proliferation (Porrello et al. 2011, 2012). In contrast, P7 rat hearts, where nearly all cardiomyocytes have already undergone binucleation and thus exit from a proliferative state, are unable to regenerate. This supports the current view that permanent exit from a proliferative state coincides with binucleation (reviewed in Sect. 2.1).

In contrast to mammalian cardiomyocytes, adult zebrafish cardiomyocytes proliferate in response to body size-related increased hemodynamic demand (Wills et al. 2008) as well as injury models such as cell ablation (Wang et al. 2011), apex resection (Poss et al. 2002) and cryoinjury (Schnabel et al. 2011). Thus, zebrafish cardiomyocytes maintain proliferative-competence throughout adulthood.

Collectively, these observations along with those in Sect. 4.1 suggest that the regenerative capacity of the mammalian heart may be dependent upon the proportion of cardiomyocytes actively undergoing proliferation, and not due to de novo activation of non-cycling cardiomyocytes. However, even though the regenerative capacity of mammalian fetal and neonatal hearts is, from a developmental perspective, not surprising it might help to understand why the adult mammalian heart cannot regenerate and thus provide new insights whether it is possible to restore proliferative-competence.

4.3 Cardiomyocyte Migration in Regeneration

A potential clue as to why the mammalian fetal heart loses regenerative capacity as gestation progresses may be related to not only reduced cellular proliferation but cell migration as well. For example, it has recently been shown that cardiac regeneration in zebrafish requires cardiomyocyte migration to the injury site (Itou et al. 2012).

The migratory ability of mammalian cardiomyocytes has not been thoroughly investigated. However, there is clear evidence that migration occurs not only during zebrafish but also during mammalian fetal development. Growth of the trabecular layer in both mammals and zebrafish involves cardiomyocyte migration from the compact layer (Toyofuku et al. 2004; Liu et al. 2010a). The regulation of mammalian cardiomyocyte migration is poorly understood. However, it has been shown that cardiomyocytes in the trabecular layer express $p57^{KIP2}$ (Kochilas et al. 1999), which in other cell-types negatively correlates with cellular migratory activity (Sakai et al. 2004). It is unknown if $p57^{KIP2}$ regulates cardiomyocyte migration from the compact layer to trabeculae during development. However, as gestation progresses, migration capacity may be down-regulated, as to control the size of the trabeculae. Should $p57^{KIP2}$ be involved in mammalian cardiomyocyte migration, its expression in cardiomyocytes in late fetal stages could reflect a differentiation phase resulting in the loss of migratory competence, in turn limiting their ability to localize to injury sites postnatally. Interestingly, the inability of mammalian cardiomyocytes to fully repair incision-induced injury in E18 fetal hearts was hypothesized to be due to reduced cardiomyocyte migratory capacity (Blewett et al. 1997).

4.4 Experimental Induction of Adult Cardiomyocyte Proliferation

The occurrence of cell cycle activity near the border zone after MI and during pathologic hypertrophy in mammals has long provided optimism that cell cycle activity can be amplified to mount a more robust regenerative response. Over the years, strategies to promote adult mammalian cardiomyocyte proliferation and cardiac regeneration include genetic modifications, ligands, and pharmacological treatments and have been reviewed extensively (Ahuja et al. 2007; Choi and Poss 2012; Engel 2005; Wadugu and Kuhn 2012; Zhu et al. 2009). Even though many of these studies are very promising there are several major issues: (i) The efficiency to induce adult cardiomyocyte proliferation is low. With regards to cell cycle entry, only activation of TWEAK/Fn14 signaling has shown to promote robust cell cycle activation with around 40 % of the cells undergoing DNA replication (Novoyatleva et al. 2010). However, TWEAK/Fn14 signaling did not promote cardiomyocytes to progress further through the cell cycle (e.g. mitosis and/or division). (ii) To date, there is no study unambiguously demonstrating that induction of adult cardiomyocyte proliferation occurs in vivo reversing cardiac damage. One intriguing study is the analysis of cardiomyocyte-specific Cyclin D2 overexpressing mice (Hassink et al. 2008; Pasumarthi et al. 2005). Long-term studies based on histological sections suggested the formation of new cardiac muscle tissue. However, tissue regeneration was not analyzed in a single heart over time; and more importantly, Cyclin D2 overexpression was established during the fetal period, which might interfere with cardiomyocyte terminal differentiation (reviewed in Sect. 2.5). This issue has to be considered as fetal c-Myc overexpression resulted in increased cardiomyocyte proliferation (Jackson et al. 1990), whereas inducible c-Myc overexpression during the adult period resulted in cardiomyocyte cell cycle-mediated hypertrophy, not proliferation (Xiao et al. 2001). Thus, it would be interesting to evaluate whether inducible Cyclin D2 transgenic adult mice are also able to regenerate after cardiac injury while applying novel unambiguous methods demonstrating in vivo regeneration kinetics. Recently, it has been shown that miR-590 and miR-199a induces regeneration after MI (Eulalio et al. 2012). However, also here the same limitations apply as there is no conclusive evidence in vitro or in vivo for adult cardiomyocyte cytokinesis, cell division and proliferation. Taken together, it appears that only a sub-population of adult cardiomyocytes has, if at all, the capacity to proliferate. It should be noted that recent studies suggest that only mononucleated cardiomyocyte may have the capacity to divide, and even then, only a fraction have this capacity, as 50 % of mononucleated cardiomyocytes that enter mitosis binucleate (Bersell et al. 2009).

Mitogenic factors such as Neuregulin may also promote cell cycle-mediated hypertrophy (Wadugu and Kuhn 2012). This observation supports concerns with regards to the use of mitotic and cytokinetic markers (e.g. phospho-histone H3 (S10) and Aurora B, respectively) to evaluate proliferation (reviewed in Sect. 3.4) as binucleation is almost undistinguishable from cell division (i.e. both processes require

chromosome segregation and furrow ingression). Indeed, many 'regenerative' mechanisms show an increase in tissue thickness. However, to what degree this is related to hypertrophy is often difficult to analyze. Thus, evidence of cardiomyocytes in mitosis and improved functional data might be misinterpreted as cardiac regeneration. Furthermore, in addition to being the favored cell-type for proliferative potential, it might be possible that mononucleated cardiomyocytes serve as a kind of hypertrophic reserve, whereby upon stress they undergo binucleation, increase in size and/or ploidy and provide additional, compensatory, contractile force to the heart.

5 Perspectives

5.1 Centrosomes: Too Much of a Good Thing?

Cells with increased ploidy are at risk for mitotic defects due to supernumerary centrosomes. Thus, this suggests a major hurdle with regards to adult heart regeneration by inducing proliferation of pre-existing cardiomyocytes, of which a substantial fraction is polyploid (reviewed in Sect. 3).

Centrosomes are the primary Microtubule Organizing Center (MTOCs) in most cell-types and are responsible for bipolar mitotic spindle formation and high-fidelity chromosome segregation. A diploid cell begins with one centrosome in G0/G1 phase. During S phase, centrosomes duplicate, such that two centrosomes are present at mitosis enabling formation of a bipolar mitotic spindle (Nigg and Stearns 2011; Tsou and Stearns 2006). In non-transformed cells, centrosome number reflects ploidy. Thus, as a tetraploid cell has two centrosomes in G0/G1, it has four centrosomes at G2/M. The presence of supernumerary (i.e. greater than two in G2/M) centrosomes is highly associated with mitotic defects (e.g. multipolar mitosis, merotelic spindle-chromosome attachments, and chromosome non-disjunction) and the generation of aneuploid cells upon division (Duensing and Munger 2002; Fujiwara et al. 2005; Ganem et al. 2007; Holland and Cleveland 2009). Due to nullsomy or gene copy number variation, aneuploid cells are generally inviable.

Given that cardiomyocytes binucleate by cytokinesis failure, G0/G1 binucleated cardiomyocytes should have supernumerary centrosomes. Thus, regeneration factors that promote mitosis and division of binucleated/tetraploid cardiomyocytes can potentially result in aneuploid cells upon division and, subsequently, cell death. While certain factors can promote binucleated cardiomyocytes to re-enter the cell cycle (Engel et al. 2005), the fate of these cells has not been evaluated to date. Certainly, a pre-requisite of any regenerative treatment should be the ability to maintain viable and stable ploidy upon cell division.

The presence of supernumerary centrosomes during mitosis does not necessarily result in mitotic defects (e.g. lowered chromosome segregation fidelity and aneuploidy). For instance, although a vast majority of tumor cells have supernumerary centrosomes, they are highly clonal with respect to their karyotype (Duesberg et al. 2011). The relatively stable transmission of ploidy in tumor cells with supernumerary

centrosomes is achieved by a phenomenon called 'centrosome clustering' whereby supernumerary centrosomes are clustered in a bipolar fashion to preserve/enable high-fidelity chromosome segregation (Kramer et al. 2011; Quintyne et al. 2005). As aneuploidy generally represents a non-viable state, centrosome clustering in transformed cells arises by Darwinian selective processes (believed to be a critical step in tumor biogenesis) (Holland and Cleveland 2009). Given that adult polyploid cardiomyocytes do not normally divide would suggest they lack any intrinsic ability to cluster supernumerary centrosomes. In this regard closer inspection of postnatal cardiomyocyte metaphases in the literature often appear atypical. For instance, chromosomes not aligned on the metaphase plate (Engel et al. 2005), which is a characteristic of supernumerary centrosomes (Ganem et al. 2009). However, it has been described that binucleated hepatocytes are able to establish a bipolar mitotic spindle to produce two tetraploid daughter cells (Guidotti et al. 2003). This suggests that hepatocytes are capable of clustering their centrosomes in a bipolar manner. Centrosomes have yet to be investigated in cardiomyocytes and the presumed supernumerary centrosome status of binculeated cardiomyocytes presents to date a fundamental challenge towards undergoing successful division and the formation of viable daughter cells.

5.2 Absence of Cancer in the Heart

To date, adult cardiomyocyte-born tumors are extremely rare (Dell'Amore et al. 2011). Why cardiomyocytes are highly resistant to tumorigenesis is unclear. Whereas mutations of p16INK4a are highly associated with tumorigenesis, mutations in the other INK4 or CIP/KIP genes do not appear to be greatly involved in the etiology of cancer (Roussel 1999). Thus, the observation that cardiomyocytes appear to primarily utilize CIP/KIP CDKI family proteins to regulate cell cycle exit (reviewed in Sect. 2.2) may offer some explanation to the relative absence of adult cardiomyocyte-born tumors. Interestingly, other cell-types that preferentially employ CIP/KIP over INK4 family members to mediate cell cycle exit (reviewed in Sect. 3.3) also rarely undergo tumorigenesis. Liposarcomas, which arise from adipocytes, are extremely rare, with an annual incidence of around 2.5 cases per million (Roh et al. 2011), and cancers derived from podocytes or vascular smooth muscle cells have to our knowledge yet to be reported. How INK4 and CIP/KIP family proteins differentially potentiate tumorigenesis is unclear.

The rare occurrence of cardiomyocyte-born tumors makes clear that it is worthwhile to study cardiomyocyte cell cycle control. Even if it should not be possible to promote cardiac regeneration the study of this topic might provide important information to control abnormal proliferation of other cell-types for preventing or treating cancer.

5.3 The Potential of Mononucleated Cardiomyocytes in Heart Regeneration

A strong positive correlation exists between the percentage of mononucleated/diploid cardiomyocytes and the regenerative capacity of the heart; 99 % of cardiomyocytes from hearts with high regenerative capacity such as newt (Matz et al. 1998), zebrafish (Kikuchi and Poss 2012), and rodent fetal and P1 neonates (Li et al. 1996; Soonpaa et al. 1996) are mononucleated/diploid, whereas only 5 % of cardiomyocytes from non-regenerative hearts, such as adult rodent and adult pig (Grabner and Pfitzer 1974; Li et al. 1996; Soonpaa et al. 1996), are mononucleated/diploid. This positive correlation has led to the speculation that the adult human heart, where ~70 % of cardiomyocytes are mononucleated, may maintain some margin of regenerative capacity (Kajstura et al. 2012; Olivetti et al. 1996). To date, however, little evidence exists that the adult human heart has any significant intrinsic regenerative capacity (Kajstura et al. 2012).

The rodent P3 neonatal heart, like many hearts with regenerative capacity, is almost entirely composed of mononucleated/diploid cardiomyocytes (Li et al. 1996; Soonpaa et al. 1996). However, when cardiomyocytes of P3 neonatal hearts cycle, unlike cardiomyocytes from hearts with regenerative capacity, they binucleate. It is fair to then speculate that binucleation or polyploidy, and not necessarily division, is a natural outcome of a cycling, mononucleated, adult mammalian cardiomyocyte. This notion is supported by the observation that 50 % of Neuregulin-treated mononucleated adult rodent cardiomyocytes that enter cytokinesis fail to undergo abscission and binucleated (Bersell et al. 2009). The significance of this observation lies in the possibility that perhaps adult human mononucleated cardiomyocytes may be similar to P3 neonatal rodent cardiomyocytes in their cycling ability. Such a position holds implications towards current models to identify heart regenerative treatments. If mononucleated adult human cardiomyocytes are similar in their cycling potential to P3 neonatal rodent cardiomyocytes, identifying factors that promote P3 cardiomyocytes to divide rather than binucleate may provide a better approach to identifying regenerative factors for the adult human heart. Some manipulations, like FGF1/p38 inhibition (Engel et al. 2005), are already capable in achieving this task. Although the efficacy of this treatment to induce cardiomyocyte proliferation in adult rodent heart injury models is modest (Engel et al. 2006a), and it is unlikely that restoration of physiological parameters is due to cardiomyocyte proliferation, it may have a more robust regenerative impact in human heart for the reasons stated above. In this sense, it would be interesting to also evaluate known pro-regenerative treatments, such as FGF1/p38 inhibition, in a P3 rodent heart resection model.

6 Summary

Traditionally, cardiomyocytes have been considered to contribute to fetal heart growth by proliferation and to pathological heart growth by hypertrophy after permanently exiting the cell cycle. However, it is clear now that the postnatal cardiomyocyte is capable of entering the cell cycle, undergoing a dynamic range of outcomes in response to various experimental, pathophysiological, and homeostatic conditions. Understanding the underlying mechanisms responsible for the different outcomes of cardiomyocyte cell cycle activity may provide new opportunities to treat disease.

In order to understand cell cycle control in cardiomyocytes it appears important to first elucidate the mechanisms that regulate cardiomyocyte cell cycle exit after birth and to determine whether this process is reversible. With regards to cell cycle exit, the most prominent events associated with the transition of cardiomyocytes from a hyperplastic to a hypertrophic state are binucleation and the loss of "fetal gene expression". Therefore, these characteristics were used to describe the post-mitotic state as terminally differentiated state. How proliferation and differentiation are interlinked remains unclear. However, accumulating studies suggest that the fetal cardiomyocyte must exit the cell cycle before being able to acquire differentiation characteristics of an adult cardiomyocyte (reviewed in Sect. 2.5).

In the past few decades the perception of cardiomyocytes being permanently withdrawn from the cell cycle has changed dramatically. It is now appreciated that cell cycling can occur in adult cardiomyocytes. Initially, cycling during pathological hypertrophy has been interpreted as solely hyperplasia as part of an endogenous attempt to regenerate (Beltrami et al. 2001; Meckert et al. 2005). While this hypothesis is still unproven and controversial, it is now established that cell cycle entry of cardiomyocytes represents also the program of pathological hypertrophy (i.e. cell-cycle dependent hypertrophy) (reviewed in Sect. 3.1). In addition, several studies have provided evidence that it is possible to induce at least a subset of adult cardiomyocytes to undergo proliferation (reviewed in Sect. 4.4). This issue calls into the question in what state of the cell cycle do postnatal cardiomyocytes reside and are there possibly populations of different states? In the most classical terms, the majority of postnatal mammalian cardiomyocytes does not appear to be senescent or terminally differentiated as they can re-enter the cell cycle and re-express the fetal gene program, but yet they are not quiescent as they cannot readily divide. Thus, cardiomyocytes represent a quasi-quiescent state. The preference of CIP/KIP CDKIs to regulate cell cycle exit and the fact that other hypertrophic cell-types share this similarity may come to define a new state of cell cycle exit (reviewed in Sect. 3.3).

A critical question in terms of cardiac regeneration is whether the cell cycle-related changes that occur postnatally can be reversed to promote cardiac regeneration by cardiomyocyte proliferation. One concept to approach this issue is the hypothesis that induction of cardiomyocyte proliferation requires dedifferentiation. However, recent data suggest that is not a prerequisite, but rather a consequence of cardiomyocytes entering the cell cycle (reviewed in Sect. 3.5). Another hypothesis is that a

sub-population of cardiomyocytes does not undergo differentiation and remains in a fetal-like state (reviewed in Sect. 3.4). Unfortunately, to date there is no marker existing that enables us to predict whether a cycling cardiomyocyte divides or undergoes polyploidization.

The general consensus is that cardiomyocytes differentiate to a more highly specialized cellular state during the perinatal period. Indeed, the transition from a fetal to adult gene program appears to be designed to accommodate energy and hemodynamic demands of the postnatal environment. However, as differentiation and proliferation are not mutually exclusive in cardiomyocytes, it is difficult to define "one" differentiation state. In fact, there is no evidence that any of the differentiation characteristics like adult gene program, binucleation or sarcomeric organization are inconsistent with the ability to proliferate. Thus, it appears unlikely that markers defining these "classical" differentiation characteristic will help to predict cycling outcomes. On the other hand, there is evidence that cell cycle exit is necessarily required for proper function of the adult heart. For example adult hearts with cardiac-specific overexpression of CDK2 continue to express the fetal gene program, without observable decline in function relative to wild-type littermates under physiological conditions (Liao et al. 2001). What then does it mean to undergo differentiation? It appears plausible to define the onset of contraction during early fetal development (which begins shortly after cardiogenesis) as differentiation. However, re-expression of the fetal gene program during compensatory hypertrophy appears to be a function of cell cycle entry (reviewed in Sect. 3.5). Thus, later developmental or diseased stages should be defined rather by their cycling status. Ultimately, it appears that adult cardiomyocytes do not fit the classical definition of terminal differentiation.

Increasingly, experimental studies have shown that adult mammalian cardiomyocytes can be induced to proliferate (reviewed in Sect. 4.4). While an exciting step forward in the field of regenerative medicine, the efficiency of induction is low and translation of this knowledge to a regenerative therapy is not yet in sight. One major hurdle to progress in this field are adequate methods to detect true division, and not merely cell cycle activity and/or mitosis, as cardiomyocytes are known to endoreduplicate and bi-multinucleate.

It is important to acknowledge that cell cycle activity, or expression of cell cycle genes, has many more outcomes than cell division (Fig. 1) and to avoid common misinterpretations in the literature. Nevertheless, the available data in the literature give reason to be optimistic that cardiac regeneration through induction of cardiomyocyte proliferation is possible. However, there are still many open questions and problems to solve: (1) What is the mechanism of cardiomyocyte binucleation and polyploidization? (2) Does pathological hypertrophy represent proper cell cycle re-entry which can be built upon to promote further cell cycle progression and ultimately division? or (3) do cell cycle-associated proteins exhibit different functions in hypertrophic cells. (4) Why do hypertrophic cardiomyocytes fail to complete cell the cell cycle and divide? (5) Can all cardiomyocytes be induced to divide or is there a sub-population that possesses this ability? If so, how can such a sub-population be defined? (6) Does experimentally induced cell

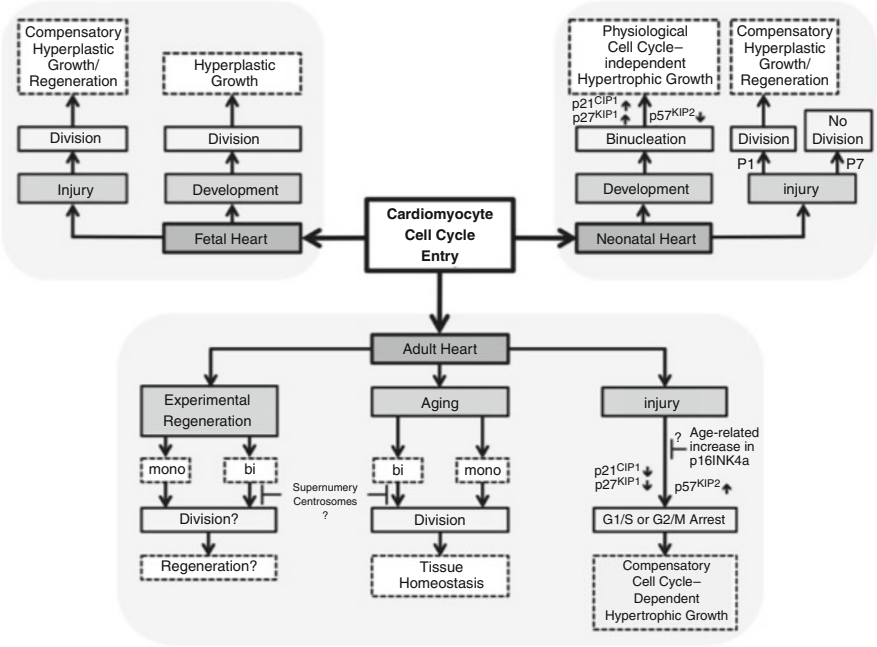

Fig. 1 Fates of cycling cardiomyocytes. Cardiomyocytes that enter the cell cycle have different fates depending on developmental stages per se and environmental cues (e.g. aging, injury, experimental regeneration). This scheme summarizes the key points of our current knowledge and identifies open questions

division produce viable progeny? (7) Does cardiac homeostasis really occur, and if so, how can a possible homeostatic process be enhanced? (8) Are there intrinsic issues preventing, at least the majority, adult cardiomyocytes from proliferating (e.g. the presence of supernumerary centrosomes or up-regulation of $p57^{kip2}$)?

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